Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A_4 production

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Regulation of 5-lipoxygenase (5LO) activity is a key determinant for the biosynthesis of proinflammatory leukotrienes. Coactosin-like protein (CLP) is an F-actin-binding protein that can also bind 5LO. Here, we report that CLP can up-regulate and modulate 5LO activity [formation of 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HPETE)], 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5-HETE), and 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTA_4) *in vitro*. Three findings are presented. First, CLP up-regulates Ca^{2+}-induced 5LO activity, in the absence of phosphatidylcholine (membrane). Apparently, CLP can function as a scaffold for 5LO, similar to membranes. Second, CLP gives a considerable (3-fold) increase in the amount of LTA_4 formed by 5LO, when present together with phosphatidylcholine. Third, CLP increases the ratio of 5-HETE/5-HPETE. These effects require protein interaction by Trp residues in ligand-binding loops of the 5LO β-sandwich; both binding and stimulatory effects of CLP were abolished for the mutant 5LO-W13/75/102A. In polymorphonuclear leukocytes stimulated with Ca^{2+} ionophore, both CLP and 5LO associated with the nucleus, whereas in resting cells, CLP and 5LO were cytosolic. These findings establish CLP as a factor relevant for 5LO product formation. Functioning as a 5LO scaffold, CLP may provide a basis for the formation of 5-HETE in the cytosol of different cell types. Furthermore, in stimulated cells, CLP appears to function in a complex together with 5LO and membranes, increasing the capacity of 5LO for leukotriene biosynthesis.

arachidonic acid | eicosanoid | inflammation

The two initial steps in leukotriene (LT) biosynthesis are catalyzed by 5-lipoxygenase (5LO), i.e., 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 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid (LTA_4; ref. 1). The actions of LTs as inflammatory mediators may be most recognized in relation to asthma, but several findings now imply a role for 5LO and LTs in another chronic inflammatory disorder, i.e., atherosclerosis (2, 3). Recent studies also support a role for 5LO metabolites in cancer cell survival (4–6).

A model of the 5LO structure, based on the crystal structure of the ferrous form of rabbit reticulocyte 15-lipoxygenase (7), consists of an N-terminal β-sandwich (residues 1–114) and a larger C-terminal catalytic domain containing prosthetic iron (residues 121–673; for review, see ref. 8). Several factors influence 5LO enzyme activity. Ca^{2+} binds to the C2-like β-sandwich (9, 10), leading to association with the nuclear membrane and increased enzyme activity. Recently, various glycerols were also reported to activate 5LO *in vitro*, by the N-terminal β-sandwich (11). MAP kinase pathways can activate 5LO during cell stress (8, 12), whereas phosphorylation by protein kinase A inhibited 5LO activity (13). ATP is another factor stimulating 5LO activity; the binding site remains undefined. Lipid–OOH oxidizes iron in 5LO to the active ferric form (14). In addition to the provision of substrate and promotion of an active 5LO protein conformation, activation mechanisms may facilitate contact with lipid–OOH (15).

Human coactosin-like protein (CLP) is similar to *Dictyostelium discoideum* coactosin (16), a member of the ADF/cofilin group of actin-binding proteins. We identified human CLP as a 5LO-binding protein by using a yeast two-hybrid system with 5LO as bait (17). Binding was confirmed for both human and mouse CLPs (18, 19). Human CLP also binds F-actin and was found to colocalize with actin stress fibers in transfected CHO and COS-7 cells (20). Mutagenesis showed the involvement of CLP Lys-75 and -131 in binding to F-actin and 5LO, respectively (18). In the CLP structure (21) K75 and K131 are close, indicating overlapping binding sites.

Here we report that CLP can serve as a scaffold for Ca^{2+}-induced 5LO activity. When CLP was present (no membrane added), 5-HPETE was formed. When both CLP and phosphatidylcholine (PC) were present in the *in vitro* assays, LT formation increased considerably. Similar to 5LO, CLP was found to associate with the nucleus after stimulation of human polymorphonuclear leukocytes (PMNL) with Ca^{2+} ionophore A23187.

Results

CLP Supports Ca^{2+}-Induced 5LO Activity and Increases the Formation of LTA_4. The effect of CLP on the formation of 5-HPETE, 5(*S*)-hydroxy-6-*trans*-8,11,14-cis-eicosatetraenoic acid (5-HETE), and LTA_4 (measured as 6-*trans*-LTB_4 and 12-epi-6-*trans*-LTB_4) was determined by HPLC. A representative set of data illustrating the effects of CLP added in stoichiometry 1:1 to wild-type 5LO, at different concentrations of PC, is shown in Fig. 1*A*. From these activity data, three observations are made.

First, CLP increases the 5LO total product formation (white bars in Fig. 1*A*). When no PC or CLP was present, total 5LO products were low (3.6 μmol/mg). However, in the presence of CLP, the total amount of 5LO products formed (21.7 μmol/mg) was almost the same as in presence of 25 μg/ml PC (25.7 μmol/mg). When CLP was added together with a lower amount of PC (2.5 μg/ml), total 5LO products increased 3.8-fold, from 8 to 30 μmol/mg. When CLP was added together with PC at the intermediary concentration (10 μg/ml), the increase was $\approx$2-

Conflict of interest: No conflicts declared.

Abbreviations: 5-HPETE, 5(*S*)-hydroperoxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 5-HETE, 5(*S*)-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid; 13-HPODE, 13(*S*)-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid; 5LO, 5-lipoxygenase; AA, arachidonic acid; CLP, coactosin-like protein; LT, leukotriene; PC, phosphatidylcholine; wt-5LO, wild-type 5LO; PMNL, polymorphonuclear leukocyte; LTA_4, 5(*S*)-*trans*-5,6-oxido-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid.

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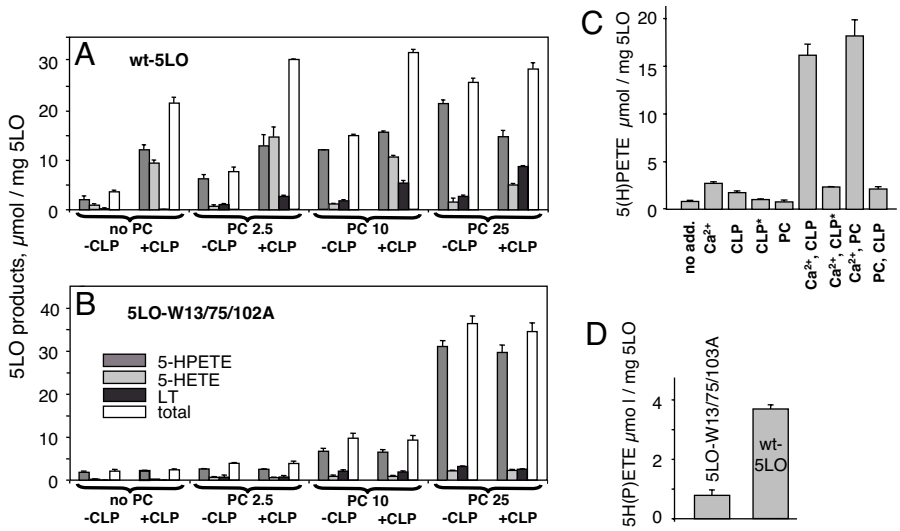

Fig. 1. Formation of 5LO products by wt-5LO in the presence of CLP and/or PC. The HPLC assay is described in *Materials and Methods.* Results are mean ± SE ($n = 3$). (*A*) Purified recombinant wt-5LO was incubated for 10 min at room temperature in the presence of AA (100 μM), Ca^{2+} (100 μM), ATP (1 mM), and 13-HPODE (10 μM). CLP (stoichiometry 1:1) and PC (μg/ml) were added as indicated. Bars correspond to 5-HPETE, 5-HETE, LT, and total products, as indicated in *B*. (*B*) Formation of 5LO products by 5LO-W13/75/102A in the presence of CLP and/or PC. Same as in *A*, with 5LO-W13/75/102A. (*C*) The effect of CLP on 5LO activity requires Ca^{2+}; heat-treated CLP (CLP*) is ineffective. The formation of 5-HPETE determined by HPLC assay, in the presence of AA (100 μM), ATP (1 mM), and 13-HPODE (10 μM). Ca^{2+} (100 μM), CLP (stoichiometry 1:1), heat-treated CLP (65°C, 10 min), and PC (25 μg/ml) are added, as indicated. Results are mean ± SE ($n = 3$). (*D*) The activity of mutant 5LO-W13/75/103A (relative to wt-5LO) is more reduced at low concentrations of AA and PC. Formation of 5-HPETE determined by HPLC assay, in the presence of AA (10 μM), PC (2.5 μg/ml), Ca^{2+} (100 μM), ATP (1 mM), and 13-HPODE (10 μM). Results are mean ± SE ($n = 3$).

fold, from 15 to 32.5 μmol/mg. At the highest PC concentration (25 μg/ml), there was only a 10% increase in total products, when CLP was also added. We conclude that CLP can replace PC, or provide an additive effect together with PC, regarding Ca^{2+}-induced 5LO activity.

Second, CLP increases LTA_4 formation (black bars in Fig. 1*A*). When no PC or CLP was present, LT production was barely detectable. Also, when only CLP was present, LT production was minute. However, when present together with PC (2.5, 10, or 25 μg/ml), CLP gave a 3-fold up-regulation of LT production as compared to PC alone. Thus, at the highest PC concentration tested (25 μg/ml), addition of CLP increased the relative amount of LTs (percentage of total products) from 11% to 31%. We conclude that CLP promotes 5LO-catalyzed formation of LTA_4, when PC is present. However, CLP alone resulted in formation of 5-HPETE but not LTA_4.

Third, in the presence of CLP, the relative amount of 5-HETE vs. 5-HPETE shifts in favor of 5-HETE. With only PC present (2.5, 10, or 25 μg/ml), 5-HPETE (dark-gray bars in Fig. 1*A*) was the dominating product, and the amount of 5-HETE (light-gray bars) was <10% of total products. However, with only CLP, or when CLP was present together with PC, the relative amount of 5-HETE increased. This was particularly clear in the absence of PC or when PC was low (2.5 μg/ml); then 5-HETEs were 41% and 48%, respectively, of total products.

In a separate experiment (Fig. 1*C*), it is shown that Ca^{2+} is required for the effects of CLP and/or PC. When 5LO-activating factors (Ca^{2+}, CLP, heat-treated CLP, and PC) were added separately, 5LO activity was low (1–3 μmol/mg). In the presence of Ca^{2+} and CLP, formation of 5-HPETE was 16 μmol/mg, as compared to 18 μmol/mg in the presence of Ca^{2+} plus PC. Heat-treated CLP (65°C, 10 min) did not support Ca^{2+}-induced 5LO activity. PC together with CLP, in the absence of Ca^{2+}, gave about the same low activity as CLP alone.

Addition of more CLP had no additional impact on the observed effects on 5LO activity. Thus, when 5LO activity was assayed (at PC 25 μg/ml) with 5LO:CLP stoichiometries, 1:2, 1:5, or 1:10, 23–25 μmol/mg 5-HPETE were formed, similar to the amount formed at stoichiometry 1:1 (24 μmol/mg). Also, the product pattern was similar in all cases. With a reduced amount of CLP was added (stoichiometry 1:0.1), no up-regulation of 5LO activity was detected.

Trp Residues in 5LO Required for the Effects of CLP on 5LO Activity. Three Trp residues in the ligand-binding loops of the 5LO C2-like domain (Trp-13, -75, and -102) were previously found important for binding of the isolated 5LO C2-like domain to PC (10). It appeared that the effects of CLP on 5LO activity were similar to those of PC (membrane). The triple mutant 5LO-W13/75/102A was expressed, and activity assays were performed. We were surprised to find that this mutant has good Ca^{2+}-induced activity in the presence of PC (Fig. 1*B*). However, all three effects of CLP on 5LO activity (formation of 5-HPETE, more LTA_4 in the presence of PC, increased 5-HETE/5-HPETE ratio) are absent (compare Fig. 1 *A* and *B*).

Thus, it was unexpected to find that, when incubated with PC (25 μg/ml) and AA (100 μM), the total activity of the triple mutant (5LO-W13/75/102A) was actually higher than for wild-type 5LO (wt-5LO). However, at lower PC (10 or 2.5 μg/ml), the triple mutant had slightly reduced activity (50–70% of wt-5LO; Fig. 1 *A* and *B*). When the concentration of AA was also reduced (to 10 μM), a more pronounced reduction of activity was observed for this mutant, to ≈25% of wt-5LO (Fig. 1*D*). Thus, at low PC and AA, the contribution of Trp-13, -75, and -102 to the efficiency of the 5LO reaction is clear. Apparently, the three Trp residues are not essential for the effect of PC on 5LO activity and thus possibly not for membrane binding of intact 5LO, although they were crucial for binding of the isolated 5LO β-sandwich to PC (10). In contrast to this gradual effect on PC-supported 5LO catalysis, the three Trp residues are essential for the effects of CLP (Fig. 1 *A* and *B*).

Binding of CLP to 5LO-W13/75/102A. Because CLP did not support activity for the mutant 5LO-W13/75/102A, we tested whether this 5LO mutant could bind CLP by pull-down assay. As found before (18), 5LO was bound to GST-CLP (attached to GSH-

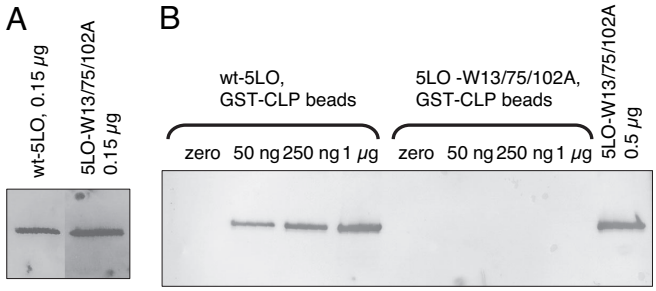

Fig. 2. Mutant 5LO-W13/75/103A does not bind GST-CLP. (*A*) The similar immunoreactivities of wt-5LO and 5LO-W13/75/103A (on the same gel) are shown. (*B*) A GST pull-down experiment, performed as described in *Materials and Methods*, is shown. Aliquots of bead eluates were analyzed for 5LO by Western blots.

agarose beads) in a dose-dependent manner but not to GST. However, no association of 5LO-W13/75/102A to GST-CLP was detectable (Fig. 2).

CLP Mutant Lys131Ala Has No Effect on 5LO Activity. In previous studies, it was shown that the mutant CLP-K131A displayed clearly reduced binding to 5LO but could still bind F-actin. On the other hand, CLP-K75A could bind to 5LO but not to F-actin (18, 20). Here, the mutant CLP-K131A did not give any of the three effects on 5LO activity (formation of 5-HPETE, more LTA_4 in presence of PC, increased 5-HETE/5-HPETE ratio), whereas CLP-K75A did (Table 1). Thus, Lys-131 in CLP is important not only for binding to 5LO but also for the effects of CLP on 5LO enzyme activity.

CLP Increases the Initial Velocity of 5LO Catalysis. Time courses of formation of 5-HPETE were monitored. In the cuvette assay incubations, [Ca^{2+}] was 100 μM, whereas concentrations of AA (10 or 20 μM) and 13(*S*)-hydroperoxy-9-*cis*-11-*trans*-octadecadienoic acid (13-HPODE; 2 μM) were lower than for the HPLC assay, and ATP was absent. The appearance of the time courses was the same in the presence of PC or CLP, with a high initial reaction velocity, which starts to level out after 1–2 min (Fig. 3*A*). Both the initial velocity of catalysis and the final amount of 5-HPETE were higher in presence of CLP than with PC (10 μg/ml). The highest activity was observed when both CLP and PC were added, and activity was very low in the absence of PC/CLP. The same pattern was seen at AA concentrations 10 and 20 μM (see Table 2). These results show that CLP is effective also at low AA concentrations, below the critical micellar concentration.

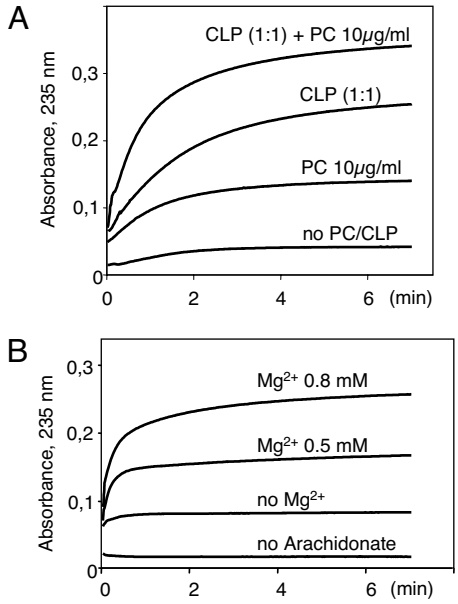

Fig. 3. Time courses of 5LO activity. The spectrophotometric assay is described in *Materials and Methods*. The reaction volume was 500 μl, and the concentration of AA was 20 μM. (*A*) Ca^{2+} (100 μM) induced formation of 5-HPETE by 0.5 μg of 5LO, in the presence of PC (10 μg/ml) and/or CLP (stoichiometry 1:1). (*B*) Mg^{2+} (0.5/0.8 mM) induced formation of 5-HPETE by 0.3 μg of 5LO, in the presence of CLP (stoichiometry 1:1).

CLP Increases 5LO Activity in the Presence of Mg^{2+}. Mg^{2+} (at mM concentrations) can activate 5LO *in vitro* in the presence of PC (22), by ligand-binding loops in the C2-like domain (9). As shown in Table 1, CLP can support Mg^{2+}-induced activation of 5LO. All three effects of CLP were observed, i.e., the formation of 5-HPETE in the absence of PC, more LTA_4 in the presence of PC, and an increased 5-HETE/5-HPETE ratio.

Time courses of 5LO activity were monitored, in the presence of CLP and Mg^{2+} (Fig. 3*B*). The general appearance was the same as with CLP and Ca^{2+}, with a high initial velocity of the

Table 1. Formation of 5LO products in the presence of CLP and CLP mutants, determined by HPLC assay (see *Materials and Methods*)

	5-HPETE, μmol/mg	5-HETE, μmol/mg	Ratio OOH/OH	LT, μmol/mg	Sum μmol/mg	LT, %
Ca^{2+} activation						
No PC/no CLP	5.5 ± 0.1	2.7 ± 0.1	2.0	0.6 ± 0.1	8.8	7
PC	17.7 ± 0.4	5.2 ± 0.1	3.4	2.5 ± 0.1	25.4	10
CLP	8.3 ± 0.6	11.1 ± 0.4	0.7	0.2 ± 0.1	19.6	1
PC+CLP	11.0 ± 0.7	12.1 ± 0.2	0.9	8.5 ± 0.2	31.6	27
CLP-K75A	0	18.1 ± 0.5	Low	1.2 ± 0.1	19.3	6
PC+CLP-K75A	3.5 ± 0.1	18.6 ± 0.5	0.2	8.3 ± 0.2	30.4	27
CLP-K131A	5.7 ± 0.5	3.3 ± 0.5	1.7	0.6 ± 0.3	9.6	6
PC+CLP-K131A	14.2 ± 0.6	6.7 ± 0.4	2.1	2.1 ± 0.4	23.0	9
Mg^{2+} activation						
No PC/no CLP	4.1 ± 0.5	1.4 ± 0.2	2.9	0.1 ± 0.03	5.6	2
PC	14.5 ± 0.3	4.7 ± 0.2	3.1	1.8 ± 0.1	20.8	9
CLP	6.1 ± 1.3	9.2 ± 1.2	0.7	0.5 ± 0.3	15.8	3
PC+CLP	11.5 ± 1.2	9.8 ± 0.4	1.2	5.7 ± 0.2	27.0	21

In the 10-min incubations, [AA] was 100 μM. Ca^{2+} (100 μM), Mg^{2+} (5 mM), PC (25 μg/ml), and CLP (stoichiometry 1:1) were added as indicated. Data are mean ± SE (n = 3).

Table 2. Initial velocities of reaction and final amount of products, determined by spectrophotometric 5LO assays

	AA, 10 μM		AA, 20 μM	
	v_{init}, μmol/mg per min	Final products, μmol/mg	v_{init}, μmol/mg per min	Final products, μmol/mg
No PC/no CLP	0.2	0.4	0.5	1.2
PC	0.8	2.1	2.2	3.2
CLP	2.4	4.6	3.6	8.0
PC + CLP	3.1	3.4	6.8	9.6

The concentration of Ca^{2+} was 100 μM. PC (10 μg/ml) and CLP (stoichiometry 1:1) were added as indicated. Data obtained with 20 μM AA are from time curves in Fig. 3*A*. v_{init}, initial velocity.

reaction, which levels out after 1–2 min. Interestingly, CLP in the presence of 0.5 or 0.8 mM Mg^{2+} gave initial reaction velocities (≈10–13 μmol/mg per min), which were higher than those obtained with 100 μM Ca^{2+} (3.6 μmol/mg per min; see Table 2).

Apparent Peroxidase Activity of CLP Together with 5LO. From the data above (Fig. 1 *A* and *B*; Table 1), it is clear that the combination of CLP and 5LO promotes conversion of 5-HPETE, formed by 5LO, to 5-HETE. To test this more directly, 5LO and/or CLP was incubated with 25 μM 5-HPETE, in the presence of Ca^{2+} (0.1 mM) and 13-HPODE (10 μM). No PC or AA were included; also, no reducing agents were present. The findings (Fig. 4) support that the combination of 5LO and CLP can reduce exogenous 5-HPETE and also 13-HPODE. CLP or 5LO alone is not as effective. Because 13-HPODE was also reduced to a similar extent, this may indicate that CLP promotes reduction of lipid–OOH bound to a proposed second fatty acid-binding site on 5LO (23, 24).

Subcellular Localization of CLP in Human PMNL. Leukocytes (mainly PMNL) were prepared from human buffy coats. Cells were incubated in the presence or absence of Ca^{2+} ionophore A23187 (2.5 μM), lysed with Nonidet P-40, and nuclear/nonnuclear fractions were prepared. The same fractions were analyzed for 5LO and CLP by Western blots (Fig. 5). As expected, 5LO was localized in the nonnuclear fraction from unstimulated cells, whereas in cells stimulated with ionophore (3, 10, or 25 min), the major part of 5LO was present in nuclear fractions. Interestingly, CLP shows the same pattern, i.e., treatment of cells with ionophore led to the redistribution of CLP, from nonnuclear to

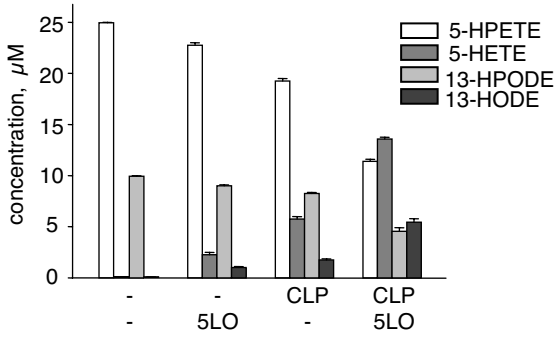

Fig. 4. 5LO and CLP reduce lipid hydroperoxides. 5LO and/or CLP (stoichiometry 1:1) were incubated with 5-HPETE (25 μM) and 13-HPODE (10 μM), according to the HPLC assay protocol. Ca^{2+} (0.1 mM) and PC (25 μg/ml) were present in all incubations, whereas AA was omitted. Results are mean ± SE (n = 2).

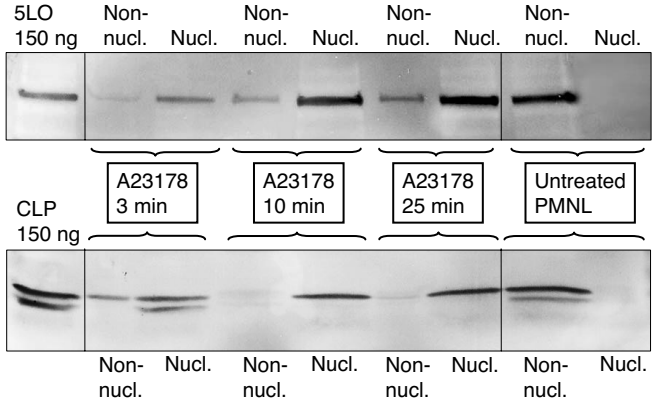

Fig. 5. Ca^{2+} ionophore A23187 induces translocation of CLP and 5LO to the nucleus in PMNL. Human leukocytes (mainly PMNL) were incubated with/without ionophore A23187 (2.5 μM) for different times and lysed with Nonidet P-40. Aliquots (35 μl) of pair-wise nuclear and nonnuclear fractions were analyzed for 5LO and CLP by Western blot.

nuclear fractions. Similar results were obtained in three experiments. In some Western blots, CLP appeared as two bands, a phenomenon that has been observed previously for recombinant mouse CLP (19). By Western blot analyses of a 10,000 × *g* supernatant from sonicated human leukocytes and known amounts of purified protein on the same membrane, amounts of 5LO and CLP were roughly estimated (not shown). The soluble protein from 1 million cells contained ≈20–50 ng of 5LO and 20–50 ng of CLP. Considering the M_r (78,000 and 16,000, respectively), on a molar basis, CLP would be approximately five times more abundant than 5LO.

Discussion

CLP was previously found to bind 5LO by coimmunoprecipitation from lysates of transfected cells. In addition, GST pull-down assay, native PAGE, and chemical crosslinking showed binding *in vitro* (18). It is well appreciated that, in the presence of Ca^{2+}, 5LO associates with membranes, leading to active enzyme (see ref. 8 for review). Here we show that CLP could replace or complement PC (membrane) as a scaffold for 5LO activity. Thus, when only CLP was present in incubations, Ca^{2+} induced a considerable formation of 5-HPETE, but LT production was minute. However, when CLP was combined with small amounts of PC, formation of both 5-HPETE and LTA_4 was increased. At the highest amount of PC tested (25 μg/ml), CLP gave a 3-fold increase in LTA_4, although there was only a slight increase in total products. CLP can bind 5LO in the absence of divalent cation (18), but it was necessary to add Ca^{2+} (or Mg^{2+}) to observe these effects on 5LO activity. Heat-treated CLP, probably with deranged structure, was not functional.

In previous studies of Ca^{2+}-induced binding of the isolated 5LO C2-like β-sandwich to PC, three mediating Trp residues (Trp-13, -75, and -102) in the ligand-binding loops were identified (10). Because it appeared that CLP and PC had similar effects on 5LO, we expressed the triple mutant 5LO-W13/75/102A. Interestingly, for this 5LO mutant, CLP did not support Ca^{2+}-induced activity, and this mutant did not bind CLP. In the same manner, we found that the mutant CLP-K131A could not support 5LO activity, in line with the previous finding that this CLP mutant had reduced binding to 5LO (18). Thus, the Trp residues in 5LO (as Lys-131 in CLP) are important both for binding of CLP to 5LO and for CLP to support Ca^{2+} activation of 5LO.

We also tested the relevance of these three Trp residues for the effect of membrane on 5LO activity. Surprisingly, the 5LO

mutant 5LO-W13/75/103A was actually more active than wt-5LO in the presence of PC (25 μg/ml) and AA (100 μM). This shows that the three Trp are not the only residues relevant for PC to support Ca^{2+}-induced activity. However, these residues clearly can contribute, because at lower added concentrations of PC (2.5 μg/ml) and AA (10 μM), the triple mutant had only 25% activity compared to wt-5LO control. These findings support the concept that residues also in the catalytic domain of 5LO may contribute to membrane association, as described for 12/15-lipoxygenase (25), and in accordance with other proteins containing a C2-domain such as cytosolic phospholipase A_2 (26).

Recent studies confirmed Ca^{2+}-induced binding of 5LO to zwitterionic PC, and it was found that this association stabilizes 5LO protein structure (27). It was also presented that 5LO can bind to other phospholipids, including cationic species. Similar to our findings for CLP, binding of 5LO to cationic phospholipid occurred in the absence of Ca^{2+}, but Ca^{2+} was required for 5LO activity. It was suggested that 5LO can bind to membranes in "productive/nonproductive modes," i.e., membrane binding *per se* may not confer 5LO activity. It appears possible that the mode of binding of 5LO also to CLP depends on the presence of Ca^{2+}, resembling the "productive/nonproductive modes" of membrane binding (27).

Our finding that CLP can replace PC as scaffold for Ca^{2+}-induced 5LO activity suggests that 5LO can be active in the cytosol without association with the nuclear membrane. Findings supporting this concept have appeared previously. Thus, in the presence of exogenous AA, 5LO products can be formed in human PMNL and Mono Mac 6 cells subjected to hypertonic buffer conditions, which prevent 5LO translocation to the nucleus (28). Also, in the B cell line BL41-E95-A, 5LO activity was high in the presence of exogenous AA, whereas association of 5LO with the nucleus was undetectable (29). CLP together with Mg^{2+} and ATP give high 5LO activity *in vitro*; these factors may thus provide a basis for active cytosolic 5LO. Such nonnuclear 5LO activity may also involve a lipid scaffold. To this end, cytoplasmic lipid bodies of eosinophils were suggested as a site for eicosanoid biosynthesis (30), and recently various glycerols were reported to activate 5LO *in vitro* (11). A putative cytosolic 5LO activity should depend on transcellular supply of AA, or that AA is provided from another cellular compartment. In either case, the S100A8/A9 complex, which can bind AA, may be relevant. This protein complex, which is very abundant in PMNL, was suggested to function as an intermediate AA reservoir, capable of delivering AA to metabolizing enzymes hours after phospholipid hydrolysis (31). Furthermore, it was suggested that 5LO may be in different cellular loci when exogenous/endogenous AA is metabolized (32).

In the established model for cellular LT biosynthesis, 5LO and cytosolic phospholipase A_2 migrate from the cytosol to the nuclear membrane when the cell is activated and catalysis occurs (33). Our data indicate that CLP up-regulates LT formation when 5LO is active at the nuclear membrane. When Ca^{2+}-induced activity was supported by CLP and PC together, the highest formation of LTA_4 was obtained (Fig. 1*A*). Furthermore, in PMNL stimulated with ionophore, both CLP and 5LO became associated with the nuclear fraction (Fig. 5). These findings suggest that 5LO can form a complex including both CLP and membrane (PC). Apparently, formation of LTA_4 is favored by such a three-partner complex. Previous *in vitro* studies showed that the relative concentrations of AA and PC in assays influenced conversion of AA to LTA_4. Also, an increased concentration of 5LO gave more LTA_4, and in transfected cells, the presence of 5LO-activating protein (FLAP) increased LTA_4 formation (reviewed in ref. 34). Cytosolic CLP (142 residues) and permanently membrane-bound FLAP (161 residues) are similar in size, but there are no striking sequence similarities. In intact cells, LTA_4 can be the major 5LO product (35); apparently, CLP is one of the factors promoting LTA_4 formation.

Three Trp residues in the 5LO C2-like domain (W13, W75, and W102) were required for binding of CLP and CLP support of Ca^{2+}-induced 5LO activity. This indicates a hydrophobic interaction, in line with the previous observation that high salt promoted binding between mouse CLP and 5LO (19). One could visualize a complex where 5LO binds CLP, and membrane is bound by residues in both 5LO and CLP. A precedent for such a three-partner complex is the association of pancreatic lipase, colipase, and membranes (36). Inactive lipase binds to the relatively small colipase (95 aa) by its C-terminal β-sandwich, whereas contact with a lipid–water interphase generates an active form where colipase also contacts residues in the lipase catalytic domain, and "opening of a lid" permits access to the active site. In the active complex, residues in both lipase and colipase contribute to micelle binding. Interestingly, the C-terminal β-sandwich of pancreatic lipase is virtually identical to the N-terminal β-sandwich of rabbit 15-lipoxygenase and thus very similar to the 5LO C2-like domain. When this identity was noticed, it was suggested that FLAP could bind 5LO, similar to the binding between colipase and lipase (7). Binding between 5LO and FLAP has not been demonstrated (37), but 5LO binds CLP by its N-terminal C2-like β-sandwich, leading to up-regulation of activity. When AA is converted to 5-HPETE and further to LTA_4, 5LO performs hydrogen abstractions at carbons 7 and 10. We speculate that association of 5LO with both CLP and membrane confers an active 5LO conformation, which promotes the second hydrogen abstraction (at C-10), leading to LTA_4 formation.

In addition to the effects of CLP on 5LO activity, we also found that CLP stabilizes 5LO, reducing nonturnover inactivation (unpublished data). Thus, CLP may function as a chaperone and/or scaffold for 5LO, and it seems possible that in the cell, 5LO is always in complex with CLP. According to our *in vitro* data, when activated by Ca^{2+} or Mg^{2+}, this complex is capable of producing 5-HPETE. Formation of LTA_4 is determined by the well established translocation of 5LO to the nuclear membrane; CLP may comigrate with 5LO in this translocation. In incubations where both CLP and PC were present, the highest yield of LTA_4 was obtained; thus CLP and PC do not compete, but rather function together to improve the capacity of 5LO for LT biosynthesis. Pharmacological intervention with the CLP–5LO association may be a novel strategy to reduce LT formation in the cell.

Materials and Methods

Expression of CLP and 5LO. Recombinant human CLP was expressed as GST fusion protein, using the plasmid pGEX-5X-1-CLP (18, 21). The fusion partner was removed by digestion with factor Xa and anion exchange chromatography on MonoQ. Recombinant human 5LO was expressed from the plasmid pT3–5LO and purified on ATP-agarose [Sigma (St. Louis, MO) A2767; refs. 9 and 38]. The plasmid 5LO-W13/75/102A was constructed from pT3–5LO by three consecutive mutations, using the QuikChange kit (Stratagene). The mutated 5LO protein was expressed at 27°C, as wt-5LO.

HPLC Assay of 5LO Enzyme Activity. Incubations were performed in Eppendorf tubes at room temperature for 10 min. First, buffer AB (50 mM Tris·Cl pH 7.5/2 mM EDTA) was added to tubes, followed by substrate mix containing Tris·Cl, pH 7.5, $CaCl_2$, $MgCl_2$, PC (Sigma P-3556), AA (Nu-Chek Prep, Elysian, MN), 13-HPODE, and ATP. For mixing, tubes were immersed in a sonication bath for 1 min. The reaction was started by addition of 5LO (100–250 ng) dissolved in buffer AB. CLP (stoichiometry 1:1) was added to the 5LO solution (in buffer AB) 10 min before incubations and thus added to incubations together with 5LO.

Final concentrations in the 100-μl incubation volume were: 77.4 mM Tris·Cl, pH 7.5/1.2 mM EDTA/10 μM 13-HPODE/4.9 mM ATP. Ca^{2+} (1.3 mM) and Mg^{2+} (6.2 mM) gave free ion concentrations of 100 μM and 5 mM, respectively. Concentrations of AA and PC are described in figure legends.

Incubations were terminated with 300 μl of cold-stop solution [60% acetonitrile/40% water/0.2% acetic acid (vol/vol)] containing 3.3 μM 17(*S*)-hydroxy-(13*Z*,19*Z*,15*E*)-docosatrienoic acid (internal standard at 235 nm; a kind gift from Mats Hamberg, Karolinska Institutet) and 1 μM prostaglandin B_2 (internal standard at 270 nm). 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, vol/vol) at a flow rate of 1.2 ml/min. The eluate was monitored at 235 nm for 5-HPETE and 5-HETE and at 270 nm for LTA_4 hydrolysis products. 5LO enzyme activity was calculated from the sum of peak areas, relative to internal standards.

Spectrophotometric Assay of 5LO Enzyme Activity. Incubations (total volume, 500 μl) were performed in a quartz cuvette at room temperature, and formation of products containing conjugated dienes (5-HETE and 5-HPETE) was monitored at 235 nm. The assay buffer was the same as for HPLC assay (77.4 mM Tris·Cl, pH 7.5/1.2 mM EDTA). The concentration of 13-HPODE was 2 μM, and ATP was omitted. AA (10 or 20 μM), PC (10 μg/ml), Ca^{2+}, and Mg^{2+} were added as indicated. The reaction was started by addition of 5LO (0.3–0.5 μg) or 5LO together with CLP to the sample cuvette.

GST Pull-Down Assay. Twenty micrograms of the GST-CLP fusion protein linked to glutathione-Sepharose 4B beads (20 μl of 50% slurry) was incubated with purified 5LO protein in the 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 2-mercaptoethanol). After 30-min gentle rotation at room temperature, beads were washed five times in buffer A (without BSA). Bound proteins were eluted with 200 μl of elution buffer (10 mM GSH in 50 mM Tris·Cl, pH 8.0) for 60–90 min at room temperature. Beads were sedimented, and aliquots of the supernatant eluate were assayed by SDS/PAGE and 5LO Western blot.

Subcellular Fractionation After Detergent Lysis. Human PMNL were isolated from leukocyte concentrates obtained from healthy donors at Karolinska Hospital (39). PMNL (4.5×10^7) were resuspended in 1 ml of PGC buffer (PBS containing 1 mg/ml glucose and 1 mM $CaCl_2$). Cells were preincubated for 10 min at 37°C before addition of ionophore A23187 (2.5 μM). After incubations (3–25 min) at 37°C, chilled cells were lysed with Nonidet P-40, and subcellular fractionation was performed as described (40). Aliquots (35 μl, derived from $\approx$3 million cells) of pair-wise nuclear and nonnuclear fractions were immediately mixed with 7 μl of concentrated SDS loading buffer, heated for 5 min at 95°C, and analyzed for 5LO and CLP by SDS/PAGE and immunoblotting as described (9, 15, 18). In-house antisera for 5LO (1551) and CLP were used. Lamin B present in the nuclear membrane was used as a control for the fractionation procedure (antibody from Santa Cruz Biotechnology, Santa Cruz, CA).

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