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Distinct parts of leukotriene C_4 synthase interact with 5-lipoxygenase and 5-lipoxygenase activating protein

Tobias Strid[#], Jesper Svartz[#], Niclas Franck, Elisabeth Hallin, Björn Ingelsson, Mats Söderström, and Sven Hammarström[*]

Division of Cell biology, Department of Clinical and Experimental Medicine, Linköping University, SE-58185 Linköping, Sweden

**Corresponding author:*

Sven Hammarström

Division of Cell Biology

Department of Clinical and Experimental Medicine

Linköping University

SE-58185 Linköping, Sweden

Tel: +46-13-224277; Fax: +46-13-224149

Email: sven.hammarstrom@liu.se

[#] These authors contributed equally

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Abstract

Leukotriene C_4 is a potent inflammatory mediator formed from arachidonic acid and glutathione. 5-lipoxygenase (5-LO), 5-lipoxygenase activating protein (FLAP) and leukotriene C_4 synthase (LTC_4S) participate in its biosynthesis. We report evidence that LTC_4S interacts *in vitro* with both FLAP and 5-LO and that these interactions involve distinct parts of LTC_4S. FLAP bound to the N-terminal part / first hydrophobic region of LTC_4S. This part did not bind 5-LO which bound to the second hydrophilic loop of LTC_4S. Fluorescent FLAP- and LTC_4S-fusion proteins co-localized at the nuclear envelope. Furthermore, GFP-FLAP and GFP-LTC_4S co-localized with a fluorescent ER marker. In resting HEK293/T or COS-7 cells GFP-5-LO was found mainly in the nuclear matrix. Upon stimulation with calcium ionophore, GFP-5-LO translocated to the nuclear envelope allowing it to interact with FLAP and LTC_4S. Direct interaction of 5-LO and LTC_4S in ionophore-stimulated (but not un-stimulated) cells was demonstrated by BRET using GFP-5-LO and Rluc-LTC_4S.

Cysteinyl leukotrienes are key mediators of inflammatory responses and immediate hypersensitivity reactions [1, 2]. They are generated from arachidonic acid and glutathione in activated cells of myeloid origin by reactions catalyzed by 5-lipoxygenase (5-LO) and leukotriene C_4 synthase (LTC_4S). 5-Lipoxygenase activating protein (FLAP) is structurally related to LTC_4S and facilitates the transfer of arachidonic acid released from membrane phospholipids to 5-LO [3]. Ca^{2+} activates the 5-LO [3] and causes it to translocate from cytosol and nuclear matrix to the nuclear envelope [4] where it forms the unstable epoxide leukotriene $(LT)A_4$. Addition of glutathione to LTA_4 is catalyzed by LTC_4S [5, 6]. MK886[1] prevents 5-LO translocation to the nuclear envelope and inhibits LT-biosynthesis in cells [7] by its tight binding to the 18 kDa membrane protein FLAP [8]. In addition to its function as an arachidonic acid carrier for 5-LO [3, 9] FLAP has been postulated to serve as an anchor for 5-LO at the nuclear envelope following cell activation. It is likely that interactions at the nuclear envelope between components of the leukotriene biosynthetic complex are crucial for the control of leukotriene synthesis. LTC_4S has previously been shown to form homo-oligomers in living cells [10] but also to interact with FLAP [11] and microsomal glutathione S-transferase 1 (MGST1) [12]. The crystal structures of MGST1, LTC_4S and FLAP have shown strong similarities including homotrimeric structures for all three proteins [13-16].

Materials and Methods

Materials. Vectors encoding GFP (pGFP^2-C1-C3) or *Renilla* luciferase (p*R*lucC1-C3) and the luciferase substrate coelenterazine (DeepBlueC™), were from BioSignal Packard (Montreal, Canada), monoclonal GFP antibody (B-2) from Santa Cruz Biotechnology (Santa Cruz, CA), 5-LO antibody from Research Diagnostics (Flanders, NJ) and Renaissance® Western blot

[1] 3-(1-(p-Chlorobenzyl)-5-(isopropyl)-3-tert-butylthioindol-2-yl)-2,2-dimethylpropanoic acid

chemiluminescence reagent from NEN Life Science Products (Boston, MA). pGEX GST-fusion protein vector, glutathione-Sepharose 4B, and ^{35}S-methionine were from Amersham Pharmacia Biotech (Uppsala, Sweden), pDsRed-C2 and pDsRed2-ER (ER-marker) from Clontech (Palo Alto, CA), SlowFade Antifade Kit and nuclear fluorescent stain ToPro3 from Molecular probes (Eugene, OR). *In vitro* transcription / translation (TNT®) kit was from Promega (Madison, WI), and SDS-PAGE molecular weight standard (broad range) from BioRad (Hercules, CA). All other materials were from sources described before [17].

Recombinant plasmids. Human 5-LO cDNA, cloned into the EGFP fusion protein vector (pEGFP-h5-LO), was a kind gift from Dr T. Izumi, Gunma University, Japan. Human FLAP cDNA cloned into a pcDNA3 expression vector was kindly provided by Dr T. Bigby, UCSD (San Diego, CA) and human 5-LO cDNA cloned into a pUC13 vector was kindly provided by Dr M. Abramovitz, Merck Frosst (Canada). Preparation of p*R*luc-hLTC$_4$S and pGFP-hLTC$_4$S (full length and truncated forms) has been described previously [10, 18]. Full length and truncated forms of LTC$_4$S were excised from the pGFP vector and subcloned in frame with GST in a pGEX vector. Full length LTC$_4$S was also subcloned into a pDsRed vector and full length human 5-LO cDNA was subcloned into pGEX and pcDNA3. Human full length FLAP cDNA was amplified by PCR and subcloned into pGFP^2-C2 and pGEX vectors. Three truncated forms of FLAP cDNA encoding amino acids 1-51, 47-119 and 107-161 were amplified by PCR (Table I) and subcloned into pGEX for production of GST fusion proteins.

GST pull-down assays. ^{35}S-Methionine-labeled 5-LO and FLAP were prepared using TNT® kit and pcDNA3-h5-LO or pcDNA3-hFLAP. GST fusion proteins were isolated from 500 ml cultures of *E.coli* Y1090 transformed with pGEX, pGEX- LTC$_4$S (or its truncated forms), pGEX-5-LO or, pGEX-FLAP (or its truncated forms). The GST proteins were immobilized on GSH-Sepharose beads and diluted in NET-N buffer (50mM Tris-HCL pH 7.4, 150 mM NaCl,

Table 1 Oligonucleotide primers for PCR

Gene sequence	Forward primer	Reverse primer
FLAP (full length)	5’-TAGAATTCATGGATCAAGAAACTGTAGGC-3’	5’-AAGCTTAGGAAATGTGAAGTAGAGG-3’
FLAP aa 1-51	5’-GTCCTGCTGGAGTTCGTGACC-3’	5’-CCGCGGAAAGGCAAGTGTTC-3’
FLAP aa 47-119	5’-CGGAATTCTTGCCTTTGAGC-3’	5’-CAGGAAGCTTATGATGCGTTTC-3’
FLAP aa 107-161	5’-GGAGAGAGAATTCAGAGCACCCCTG-3’	5’-GCAAGCTTAGGAAATGAGAAGTAGAGGG-3’

5mM EDTA and 0.5% Igepal). For binding studies 1ml aliquots of fusion protein were incubated at 4° C for 1 h with ^{35}S-labeled protein (0.05 μCi). The beads were then washed five times in NET-N buffer, boiled in SDS sample buffer and analyzed by SDS-PAGE. Radioactivity was detected by autoradiography.

Cell cultures. Human embryonic kidney (HEK) 293/T cells and COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) and split 1:5 at confluence.

Transient transfection. HEK-293/T cells and COS-7 were transfected using polyethyleneimide as previously described [17,18].

Fluorescence microscopy. HEK-293/T and COS-7 cells transfected with fluorescent fusion proteins were analyzed by fluorescence microscopy as previously described [18]. Some cells were incubated at 37 ^{o}C with 10 μM calcium ionophore A23187 for 20 min before fixing.

BRET^{2} assays. HEK 293/T cells, co-transfected with 0.5 μg of p*R*luc-LTC$_4$S and 2μg pGFP fusion constructs or pGFP, were analyzed for protein-protein interaction by BRET as described [10,18] . In some experiments, cells were incubated in serum-free medium with 10 μM A23187 for 20 min prior to detachment. Energy transfer was calculated as BRET ratio ($Emission_{515}$ – $Emission_{410}$ x C_f) / $Emission_{410}$; C_f = $Emission_{515}$ / $Emission_{410}$ for *R*luc-LTC$_4$S expressed alone. The amount of DNA in transfection experiments was equalized using empty pcDNA3 vector.

Western blots. Cells for BRET analyses were collected by centrifugation and analyzed by Western blot using anti-GFP or anti-5-LO diluted 1:200 (v/v) in 1% BSA followed by HRP-conjugated goat anti-mouse IgG antibody diluted 1:10,000 (v/v) in PBS.

Results

Interaction analyses by GST pull-down technique

GST pull-down experiments were performed using GST-fusion proteins of full length LTC_4S and seven truncated variants (1-58, 1-88, 1-115, 23-150, 57-150, 87-150, and 114-150), full length FLAP and the three truncated variants (1-51, 47-119, 107-161), and full length 5-LO. The results showed faint interaction between full length LTC_4S and 5-LO (Fig. 1B:2), while LTC_4S 23-150, 57-150 and 87-150 bound much more 5-LO (Fig. 1A:5-7 and Fig. 1B:3-5). LTC_4S 1-58, 1-88, 1-115 (Fig. 1A:2-4) showed minimal interaction and 114-150 (Fig. 1B:6) did not bind 5-LO suggesting that the second hydrophilic loop (amino acids 90-113, Fig. 1F) mediated the interaction with 5-LO when attached to hydrophobic region 3. LTC_4S also interacted with FLAP: the full length protein and truncation 1-58 bound ^{35}S-FLAP efficiently, while LTC_4S 114-150 gave less abundant interaction (Fig 1C:2-4). We also obtained clear evidence for interaction between FLAP and 5-LO: The most abundant 5-LO binding was observed for full length FLAP and truncations 1-51 and 47-119 (Fig 1 D:2-4) whereas a C-terminal domain (107-161) appeared to bind less 5-LO. Reverse experiments using GST-5-LO and ^{35}S-FLAP confirmed the interaction (Fig. 1E:2) and demonstrated that 10 μM MK886 did not diminish the binding of 5LO to FLAP. Interestingly, 10 μM LTC_4 reduced the binding significantly (Fig 1E:4) suggesting that 5-LO / FLAP interaction may serve as a feed-back control point for LTC_4 biosynthesis.

Confocal fluorescence microscopy

HEK-293/T cells, co-transfected with GFP-FLAP or GFP-LTC_4S and dsRed-ER, showed clear co-localization of these proteins at the nuclear envelope and endoplasmatic reciculum (Fig 2, left top). Similar distributions were seen using COS-7 cells co-transfected with GFP-FLAP or

GFP-LTC_4S and DsRed-ER (Fig. 2, right top). In another experiment COS-7 cells were co-transfected with GFP-FLAP and DsRed-LTC_4S (Fig. 2, bottom). Taken together the results showed that FLAP and LTC_4S co-localized not only with the endoplasmic reticulum marker but also with one another even though small areas were seen with LTC_4S without FLAP (Fig. 2, bottom).

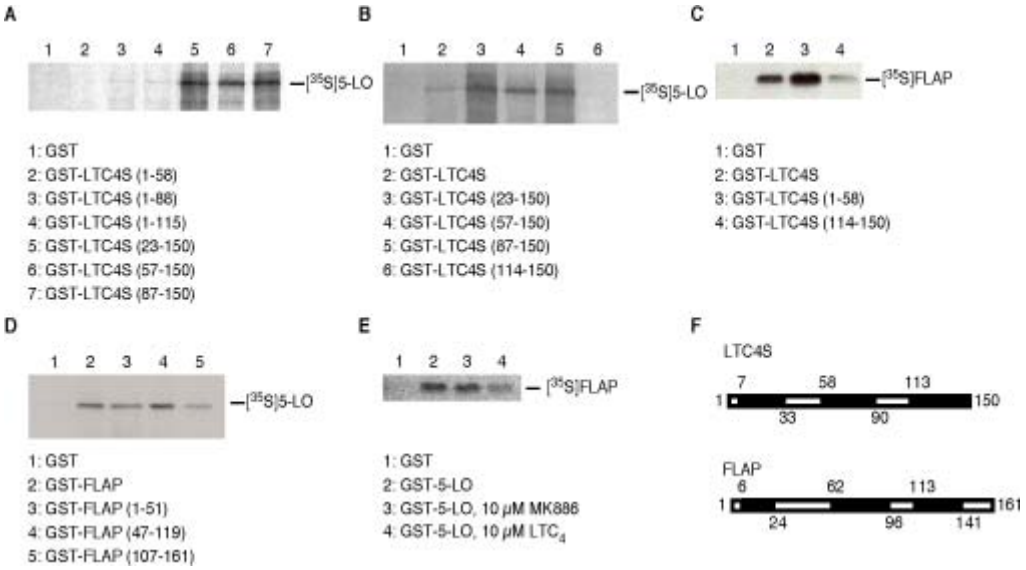

Fig. 1
A. - B. *LTC_4S interacts with 5-LO*: The largest binding of [^{35}S] 5-LO was seen with GST-LTC_4S (87-150) whereas no binding was observed with GST-LTC_4S (114-150) suggesting that the second hydrophilic loop (aa 90-113) mediated LTC_4S binding of 5-LO.
C. *LTC_4S interacts with FLAP*: The largest binding of [^{35}S] FLAP was observed for GST-LTC_4S (1-58), containing its N-terminal tail, hydrophobic domain 1, and hydrophilic loop 1. Interestingly, this part of LTC_4S did not bind 5-LO (Fig. 1A:2).
D. *FLAP interacts with 5-LO:* Truncation of GST-FLAP affected [^{35}S] 5-LO binding less than that seen for LTC_4S truncation mutants in Fig. 1 A-B.
E. *LTC_4 reduces 5-LO interaction with FLAP:* The interaction between GST-5-LO and [^{35}S] FLAP was not affected by the FLAP inhibitor MK886 but was diminished by LTC_4.
F. Depiction of hydrophilic (white) and hydrophobic (black) domains of LTC_4S and FLAP.

HEK293/T or COS-7 cells transfected with GFP-5-LO expressed most of the fluorescent fusion protein within the nuclear matrix and cytosol in non-stimulated cells (Fig. 3, row 1). After stimulation with A23187 a clear translocation of fluorescence to the nuclear envelope was

observed (Fig. 3, rows 2-3). In stimulated HEK293/T and COS-7 cells the activated 5-LO appeared mainly at the nuclear envelope and partly co-localized with the ER-marker, previously shown to co-localize with LTC_4S and FLAP.

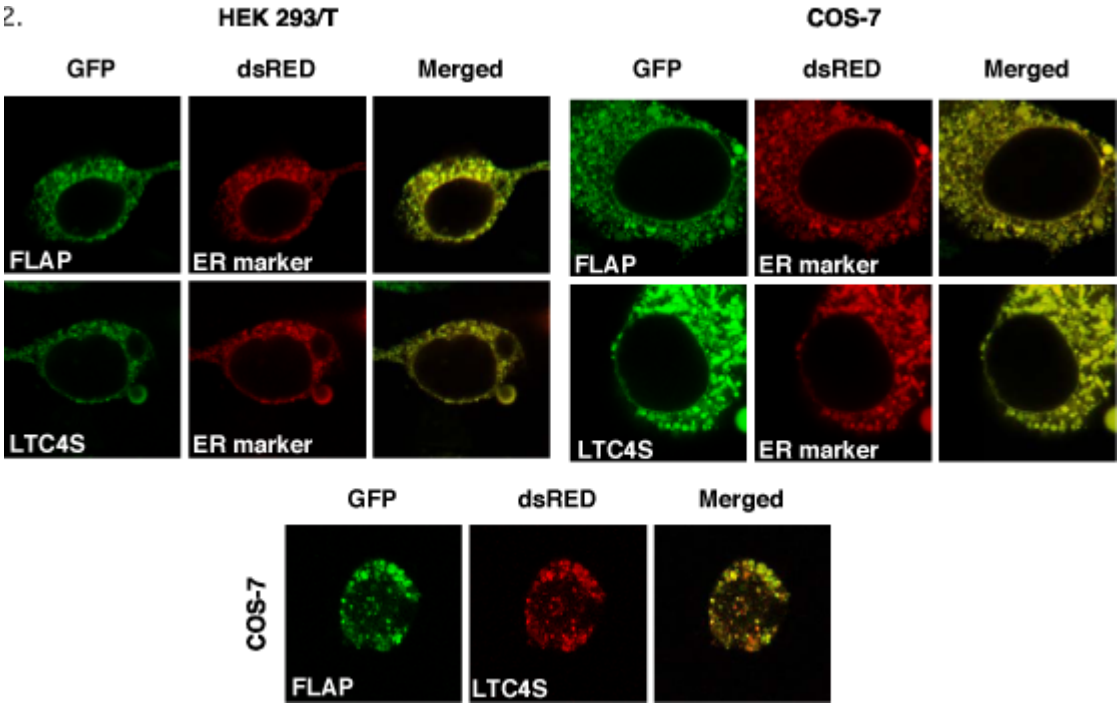

Fig. 2
FLAP and LTC_4S co-localize. HEK293/T and COS-7 cells were co-transfected with GFP-FLAP or GFP-LTC_4S and pDsRed-ER or pDsRed-LTC_4S constructs. FLAP and LTC_4S co-localized with each other and with the ER marker at the nuclear envelope.

BRET – oligomerization assay in living cells

5-LO, and LTC_4S were tested for interaction in living cells using BRET assay. Cells co-expressing *R*luc-LTC_4S and GFP-LTC_4S gave high BRET ratios (Fig. 4A) indicating that homo-oligomer formation took place as we have reported before [10]. *R*luc-LTC_4S and GFP-5-LO gave very low BRET ratios in non-stimulated cells. However, when the cells were treated with A23187, the BRET ratio increased substantially, indicating that 5-LO and LTC_4S interact physically in activated cells (Fig. 4B). As shown above ionophore stimulation caused GFP-5-LO to translocate to the nuclear envelope thus permitting its interaction with LTC_4S which is

localized there. Western blot analyses of cells from BRET experiment showed that the expression of GFP and 5-LO protein did not vary and excluded that different transfection efficiencies had influenced the BRET analyses (data not shown).

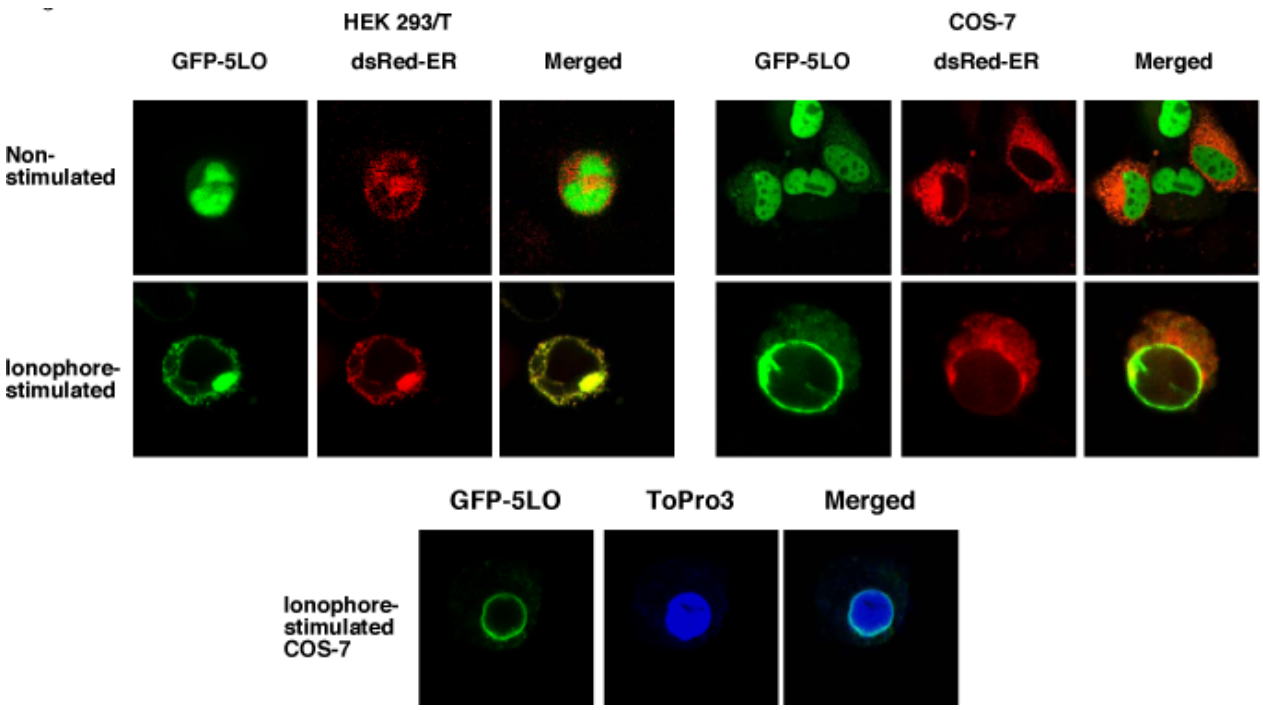

Fig. 3
5-LO translocates from nuclear matrix and cytosol to the nuclear envelope. HEK293/T and COS-7 cells were co-transfected with GFP-5-LO and pDsRed-ER. Upon activation with calcium ionophore A23187, 5-LO translocated to the nuclear envelope as shown by partial co-localization with the ER-marker. COS-7 cells were transfected with GFP 5-LO and treated with A23187 and ToPro3 nuclear stain. GFP-5-LO translocated to the nuclear envelope upon ionophore activation.

Discussion

We report here that FLAP and LTC_4S were co-localized at the ER and nuclear envelope in transfected cells and that both FLAP and LTC_4S bound 5-LO *in vitro*. 5-LO has been shown to interact with FLAP by fluorescence life time imaging microscopy and immune-precipitation but interaction with LTC_4S was not observed [11]. Abundant interaction was observed between 5-LO and both hydrophilic loops of FLAP while 5-LO bound preferentially to the second hydrophilic loop of LTC_4S. Our observation that LTC_4 reduced the interaction between FLAP and 5-LO

suggests this interaction may provide a putative site for feedback regulation of LTC_4 biosynthesis. On the other hand, FLAP inhibitor MK886 did not reduce FLAP / 5-LO interaction. We found that FLAP and LTC_4S interacted through hydrophobic regions of LTC_4S. Thus membrane spanning regions of LTC_4S trimer may contact membrane spanning regions of a neighboring FLAP trimer. The interaction was more efficient with an N-terminal part of LTC_4S than with the entire protein. It is not known if formation of LTC_4S and FLAP hetero-oligomers competes with homotrimers of each protein or if larger protein complexes are formed between homotrimers. Both hydrophilic loops of LTC_4S and FLAP point to the ER luminal side [19] suggesting that 5-LO is translocated to this side of the ER / nuclear envelope upon cell activation, a notion which is supported by our BRET experiments.

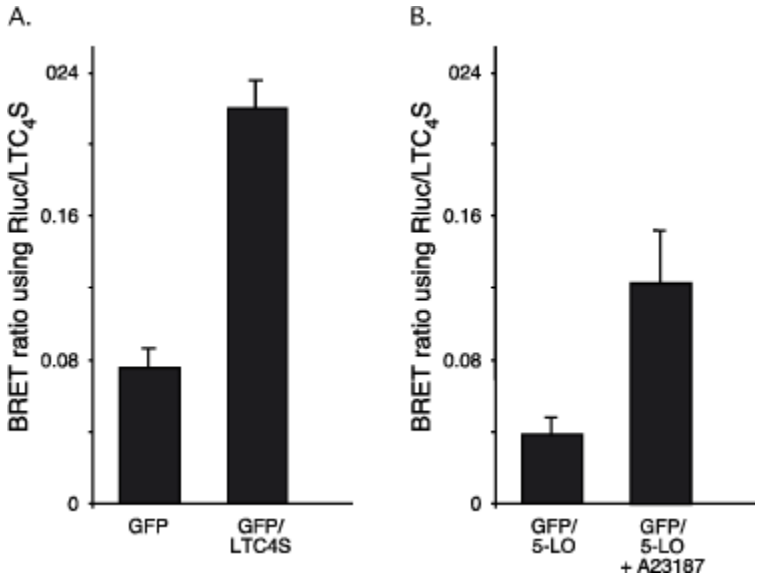

Fig. 4
*5-LO and LTC_4S interact in activated cells***.** Bioluminescence resonance energy transfer (BRET) assays were performed as previously described [10, 18]. Data are expressed as mean values ± standard deviation (n=3). For further details, see Materials and Methods. **Panel A:** homo-oligomerization of LTC_4S occurred as previously reported [10]. **Panel B**: Results indicate that 5-LO and LTC_4S interact in ionophore-stimulated but not in un-stimulated cells.

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