# A23187-Induced Translocation of 5-Lipoxygenase in Osteosarcoma Cells

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***Abstract.*** In a previous study, osteosarcoma cells expressing both 5-lipoxygenase (5-LO) and 5 lipoxygenase-activating protein (FLAP) synthesized leukotrienes upon A23187 stimulation (Dixon, R. A. F., R. E. Diehl, E. Opas, E. Rands, P. J. Vickers, J. F. Evans, J. W. Gillard, and D. K. Miller. 1990. *Nature (Lond.)*. 343:282–284). Osteosarcoma cells expressing 5-LO but not expressing FLAP were unable to synthesize leukotrienes. Thus, it was determined that FLAP was required for the cellular synthesis of leukotrienes. To examine the role of FLAP in A23187-induced translocation of 5-LO to a membrane fraction, we have studied the A23187-stimulated translocation of 5-LO in osteosarcoma cells expressing both 5-LO and FLAP, and in osteosarcoma cells expressing 5-LO only. We demonstrate that in cells expressing both 5-LO and FLAP, 5-LO translocates to membranes in response to A23187 stimulation. This 5-LO translocation is inhibited when cells are stimulated in the presence of MK-886. In osteosarcoma cells expressing 5-LO but not expressing FLAP, 5-LO is able to associate with membranes following A23187 stimulation. In contrast to the cells containing both 5-LO and FLAP, MK-886 is unable to prevent 5-LO membrane association in cells transfected with 5-LO alone. Therefore, we have demonstrated that in this cell system, 5-LO membrane association and activation can be separated into at least two distinct steps: (1) calcium-dependent movement of 5-LO to membranes without product formation, which can occur in the absence of FLAP (membrane association), and (2) activation of 5-LO with product formation, which is FLAP dependent and inhibited by MK-886 (enz me activation).

THE enzyme 5-lipoxygenase (5-LO)[1] is the first committed enzyme in the biosynthetic pathway that leads to the production of leukotrienes (1–3). Leukotrienes, produced by inflammatory cells, have potent biological effects, including leukocyte chemotaxis, bronchoconstriction, vasoconstriction, and increased vascular permeability (4). Because leukotrienes have been implicated in a variety of inflammatory diseases (5), the regulation of the synthesis of leukotrienes has been studied extensively.

5-LO has been purified, cloned, and expressed in mammalian cells (6–15). The enzyme is a 78-kD cytosolic protein with an absolute requirement for calcium and ATP for activity. Upon stimulation of 5-LO expressing myeloid cells with the calcium ionophore A23187, 5-LO translocates to a membrane compartment where it catalyzes the production of $LTA_4$ from arachidonic acid (16–19). A quantitative and temporal correlation of 5-LO translocation with leukotriene synthesis was shown in human peripheral blood leukocytes after ionophore challenge (16). It has also been demonstrated that 5-LO translocates to a membrane compartment in RBL-2H3 cells stimulated by ionomycin or the tumor promoter thapsigargin (20). In addition, two separate studies have shown translocation of 5-LO to a membrane fraction in response to receptor-mediated stimuli, namely fMLP (18) and antigen (21). In RBL-2H3 cells, it was shown that ionomycin and thapsigargin induced $LTC_4$ production and 5-LO translocation primarily by causing an influx of extracellular calcium (20). Two distinct classes of leukotriene synthesis inhibitors have been shown to selectively inhibit the A23187- and fMLP-induced translocation of 5-LO to the membrane (18, 22). These inhibitors have also been shown to interact specifically with the 18-kD membrane protein, FLAP (23). FLAP inhibitors have been shown to inhibit leukotriene synthesis in all cells that make leukotrienes and a correlation has been shown between FLAP inhibitors' potency for inhibition of leukotriene synthesis and degree of inhibition of 5-LO translocation (22). MK-886 has also been shown not only to prevent 5-LO translocation but also to reverse the translocation process (22).

To investigate the role of FLAP in leukotriene synthesis, 5-LO and FLAP were expressed in osteosarcoma cells (24). Wild-type osteosarcoma cells or cells expressing human 5-LO did not synthesize leukotrienes in response to stimulation with A23187. In contrast, cells expressing both 5-LO and FLAP synthesized leukotrienes after A23187 activation. This leukotriene production was inhibition by the compound MK-886 that binds selectively to FLAP (24). These studies

1. *Abbreviations used in this paper*: 5-LO, 5 lipoxygenase; $cPLA_2$, cytosolic phospholipase $A_2$; FLAP, 5 lipoxygenase-activating protein; GAP, GTPase activating protein; MBS, membrane binding site; NRS, preimmune serum; PKC C-2, constant region 2 of protein kinase C; TTBS, TBS + 0.05% Tween 20.

verified that FLAP is essential for leukotriene synthesis but did not define the mechanism by which FLAP activates 5-LO in osteosarcoma cells.

We utilized the osteosarcoma cell system to examine whether FLAP was essential for the membrane association of 5-LO. In the following paper, we show that 5-LO translocates to a membrane compartment in A23187-stimulated osteosarcoma cells expressing FLAP. This translocation is inhibited by MK-886, yet is not inhibited by the structurally similar indole, L-583,916 which is inactive as a leukotriene synthesis inhibitor. Unexpectedly, 5-LO also associates with membranes in A23187-stimulated cells not expressing FLAP. In contrast to cells expressing both 5-LO and FLAP, 5-LO membrane association was not inhibited by MK-886 in cells not expressing FLAP. The present experiments suggest that 5-LO translocation and activation can be considered to occur in at least two steps: (*1*) movement to membranes without product formation, followed by (2) enzyme activation resulting in product formation. Our results suggest that the first step (membrane association) is calcium dependent and can occur in cells lacking FLAP, but is inhibited by MK-886 when FLAP is present. In contrast, step 2 has an absolute requirement for FLAP and is inhibited by MK-886. We propose two possible models to suggest how this may occur within the cell. The first model does not require a direct interaction of 5-LO and FLAP, whereas the second model is based on a direct protein–protein interaction. Both models predict localization of 5-LO and FLAP in the same membrane compartment after A23187 challenge. All of the 5-LO translocation studies to date in various cell types (rat PMN, RBL-1 cells, RBL-2H3 cells, $Me_2SO$-differentiated HL-60 cells, human peripheral blood leukocytes and transfected human osteosarcoma cells) and with various stimuli (A23187, ionomycin, thapsigargin, and antigen) are consistent with either of these models.

## Materials and Methods

### Cell Culture

Human osteosarcoma cells transfected with human 5-LO cDNA have been previously described (13). These cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated FBS (Sigma Chemical Co., St. Louis, MO), 50 U/ml penicillin, 50 μg/ml streptomycin (Flow Laboratories, McLean, VA), 2 mM glutamine (Flow Laboratories), 25 mM Hepes, pH 7.4, 1.1 mg/liter sodium pyruvate (Flow Laboratories), and 200 μg/liter Gentamicin (Sigma Chemical Co.) at 37°C under an atmosphere of 5% $CO_2$. Because the expression vector for 5-LO contains the hygromycin resistance gene, 300 μg/ml hygromycin B (Calbiochem Corp., La Jolla, CA) was added to media. Cell lines transfected with the expression vector for rat FLAP contained the neomycin resistance gene as previously described (24). These cells also contained the 5-LO construct indicated above and were passaged in the same medium in the presence of both 300 μg/ml hygromycin B and 400 μg/ml G418 (Gibco Laboratories, Grand Island, NY, or Sigma Chemical Co.). Cells were grown in monolayers on 225 $cm^2$ or 600 $cm^2$ dishes (NUNC, Naperville, IL).

### Leukotriene Synthesis

Monolayers of osteosarcoma cells were rinsed with Dulbecco's phosphate buffered saline (dPBS). 1 mM stocks of MK-886 or L-583,916 were prepared in $Me_2SO$. Cells were preincubated by overlaying the monolayer with 2.5 or 6.5 ml (225 or 600 $cm^2$ plates, respectively) of dPBS containing 2 μM drug or $Me_2SO$ as control for 5 min at 37°C. Cells were stimulated by adding an equal volume of dPBS containing 2 μM A23187 in $Me_2SO$ for 10 min at 37°C. The final concentrations of drug and A23187 were 1 μM. The liquid above the cell monolayer was removed and combined with an equal volume of 0.1 nmol/ml prostaglandin $B_2$ ($PGB_2$) in ice cold methanol. Samples were acidified by addition of 10 μl of concentrated formic acid per ml of aqueous solution, extracted with chloroform and analyzed by reverse phase HPLC on a Waters C18 Novapak column, as previously described (17). Approximately 80–85% of the leukotrienes produced by these cells is secreted into the medium (data not shown). Leukotrienes were eluted with acetonitrile:methanol:water:acetic acid (28:18:54:1) pH 5.6 at a flow rate of 1.2 ml/min, monitoring absorbance at 270 nm. Identified products were 6-*trans*-$LTB_4$, 6-*trans*-12-*epi*-LTB4 and $LTB_4$, with approximate elution times of 17, 19, and 22 min, respectively. Under these conditions, $PGB_2$ eluted at 11 min. Leukotriene production was determined by comparison with the $PGB_2$ internal standard, correcting for the relative absorption maxima of the products.

### Homogenization of Cells and Preparation of Supernatants and Pellets

Immediately after stimulation of the cells and removal of the liquid above the cell monolayer, the cells were removed by scraping with a rubber spatula and pelleted at 1,000 *g* for 10 min at room temperature. Cell pellets were resuspended in homogenization buffer (50 mM potassium phosphate buffer, pH 7.1, 0.1 M NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 60 μg/ml soybean trypsin inhibitor) and disrupted by sonication as described previously (17). The samples were centrifuged at 10,000 *g* for 15 min at 4°C. The 10,000 *g* supernatants were further centrifuged at 100,000 *g* for 45 min at 4°C. The resulting 100,000 *g* supernatants (100S) were collected; the 100,000 *g* pellets (100P) were rinsed twice with KPB-2 (20 mM potassium phosphate buffer, pH 7.1 containing 2 mM EDTA and 1 mM dithiothreitol) and then resuspended in one fifth the original volume KPB-2 using a Potter-Elvehjem homogenizer.

### Broken Cell Assay of 5-LO Activity

5-LO activity was determined in 1-ml reaction volumes containing 0.1 mM arachidonic acid, 2 μM 15-hydroperoxy-11,13-eicosadienoic acid, 0.1 M Tris-HCl, 3 mM $CaCl_2$, 2 mM ATP, 1 mM dithiothreitol, 2.5% glycerol, 30 mM potassium phosphate, and ~1.5 mg 100S or 100P protein, at a final pH of 7.5. The reaction mixture was incubated for 10 min at 37°C, and the reaction was then terminated by addition of 1 ml of ice-cold ethanol containing ~2.5 nmol/ml 13-hydroxylinoleic acid as internal standard. Protein was precipitated by centrifugation at 14,000 *g* in an eppendorf microfuge for 10 min at 4°C. Aliquots (200 μl) of the resulting supernatant were analyzed by reverse phase HPLC as described previously (17). Under these conditions, the major 5-LO products are 5-HETE and 5-HPETE, which cochromatograph in our HPLC conditions. Activity is expressed as nmol 5-HETE/$10^8$ cells/10 min reaction. Similar results were obtained if activity is expressed as nmol 5-HETE/mg protein/10 min reaction.

### SDS-PAGE and Immunoblot Analysis of 5-LO

Immediately following the preparation of 100S and 100P samples, aliquots were mixed with 0.5-vol SDS sample buffer [20 mM Tris-HCl, pH 6.8, containing 0.4% (w/v) SDS, 4% glycerol, 0.24 M β-mercaptoethanol, and a trace of bromophenol blue], boiled for 5 min and subjected to SDS-PAGE on 9 × 10 cm precast 10% Tris–glycine acrylamide gels (Novex, Novel Experimental Technology, San Diego, CA) according to the method of Laemmli (25). Proteins were electrophoretically transferred to nitrocellulose membranes as described previously (26). After transfer, nonspecific sites on the membrane were blocked with 3% gelatin in TBS (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl) for 20–30 min at room temperature, and then the membranes were washed two times in TTBS (TBS + 0.05% Tween 20) for 5 min each. Blots were then incubated with a 1:200 final dilution LO32 rabbit polyclonal antiserum to purified human leukocyte 5-LO in 1% gelatin in TTBS for 2–3 h at room temperature. After washing the blot twice in TTBS, membranes were incubated with 5 × $10^5$ cpm/ml $^{125}$I-protein A (low specific activity, Du Pont–New England Nuclear) for 45 min in 1% gelatin in TTBS. The blots were washed five times in 50-mM potassium phosphate buffer, pH 7.1, 0.1 M NaCl, 0.5% Triton X-100 and exposed to XAR-5 film at −70°C for ~24 h. Autoradiographs were scanned using an LKB 2202 Ultrascan laser densitometer, and the area under the peak corresponding to the 5-LO band was used to compare relative amounts of 5-LO protein. For all conditions six to nine lanes were scanned representing two to three separate experiments with duplicate cell aliquots. Data is expressed as means ±SE. It has been shown previously by titration of purified human 5-LO into leukocyte cytosolic fractions that a linear response to increasing amounts of 5-LO can be detected by immunoblot analysis (16).

### *Immunoblot Analysis of FLAP*

100P samples from osteosarcoma cells expressing 5-LO only or 5-LO and FLAP were combined with 0.5 vol SDS sample buffer and subjected to SDS-PAGE on 9 × 10 cm precast 8–16% gradient Tris-glycine gels (Novex) according to the method of Laemmli (25). Immunoblot analysis was performed essentially as described above, except that blots were incubated with a 1:200 final dilution of preimmune serum (NRS) or H5 antiserum. H5 is a rabbit polyclonal antipeptide antibody which recognizes amino acid residues 41–52 of human FLAP.

### *Photoaffinity Labeling and Immunoprecipitation*

Photoaffinity labeling and immunoprecipitation was performed as previously described using the quindole photoaffinity ligand [$^{125}$I]L-691,678 (27). Immunoprecipitation of photoaffinity-labeled FLAP was performed with H5 antiserum according to previously described procedures (28).

### *Statistical Analysis*

All data was subjected to statistical analysis using the Student's independent *t* test (SigmaPlot 4.1; Jandel Scientific, Corte Madera, CA). Statistical significance was determined as the differences in responses between control versus challenged cells, and for cells challenged in the absence and presence of drug. An * denotes $P < 0.05\%$, ** denotes $P < 0.01$, and *** denotes $P < 0.005$.

## *Results*

### *Synthesis of Leukotrienes in A23187-Stimulated Osteosarcoma Cells Expressing 5-LO and FLAP*

Osteosarcoma cells expressing 5-LO and FLAP synthesized undetectable levels of leukotrienes in the absence of stimulation with A23187 (Fig. 1 *A*). In agreement with previous findings (24), cells stimulated with 1 μM A23187 synthesize $LTB_4$, and the nonenzymatic hydrolysis products of $LTA_4$, namely, 6-*trans* $LTB_4$ and 6-*trans*-12-*epi* $LTB_4$ (Fig. 1 *B*). These products were identified by elution position and standard ultra violet spectra identifying absorption maxima at 260, 270, and 280 nm. When cells were preincubated in the presence of 1 μM MK-886, A23187-induced leukotriene production was completely inhibited (Fig. 1 *C*). In contrast to cells preincubated with MK-886, leukotriene synthesis was not inhibited in cells preincubated with L-583,916, a compound structurally related to MK-886 but which does not inhibit leukotriene synthesis in HL-60 cells (19) (Fig. 1 *D*). We were unable to detect any leukotrienes in A23187-stimulated osteosarcoma cells expressing 5-LO only (data not shown).

### *Characterization of FLAP in Osteosarcoma Cells Expressing 5-LO and FLAP*

We wanted to verify that the osteosarcoma cells used in these experiments, transfected with both 5-LO and FLAP cDNAs, expressed full-length FLAP which was able to bind MK-886 analogs. 100,000 *g* pellets were prepared from these cells and analyzed for the presence of FLAP by immunoblot analysis and by photoaffinity labeling experiments. Fig. 2 *A* shows that FLAP can be detected in the membrane fraction of these cells by immunoblot analysis using an antipeptide antisera to FLAP. In earlier studies in sonicated rat PMN, we have shown FLAP can be found in all membrane fractions following differential centrifugation (J. Evans, unpublished data). However the highest concentration of FLAP is found in 100,000 *g* pelleted fractions (J. Evans, unpublished data). We have also investigated the presence of FLAP in un-

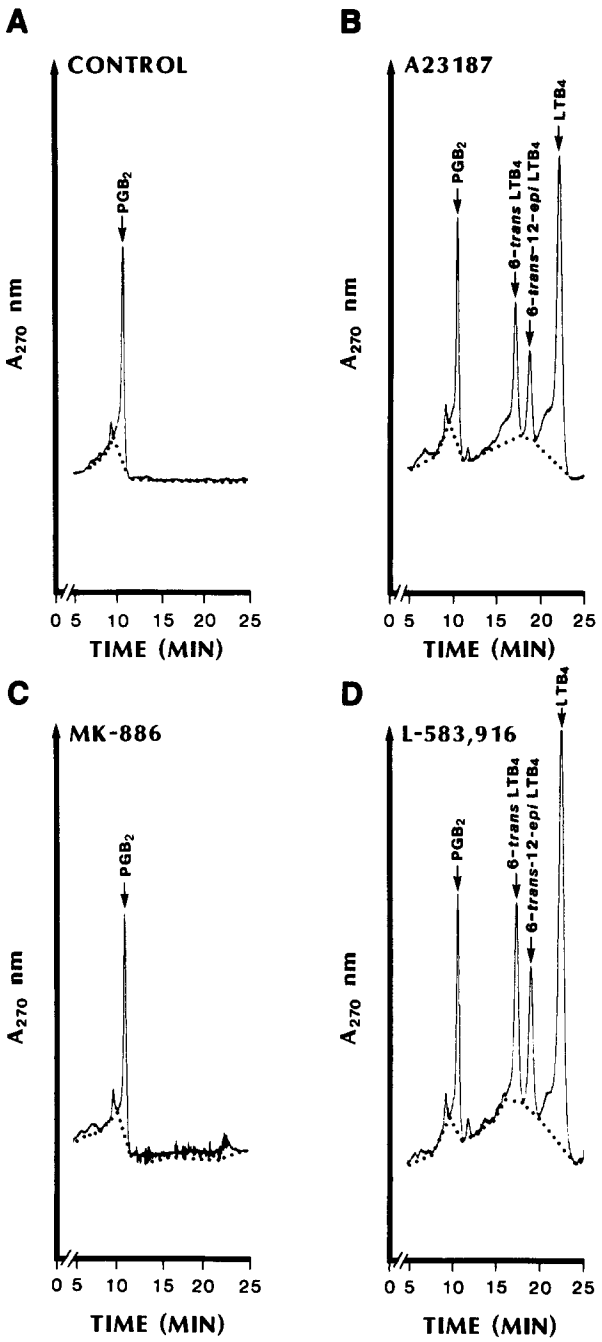


*Figure 1.* Leukotriene production by A23187-stimulated osteosarcoma cells expressing 5-LO and FLAP. Control, unstimulated cells. A23187, A23187-stimulated cells. MK-886, A23187-stimulated cells preincubated in the presence of MK-886. L-583,916, A23187-stimulated cells preincubated in the presence of L-583,916. Cell monolayers were preincubated with 1 μM MK-886 (*C*), 1 μM L-583,916 (*D*) or $Me_2SO$ as control (*A* and *B*) for 10 min at 37°C and then challenged with 1 μM A23187 (*B–D*) or $Me_2SO$ (*A*) for 10 min at 37°C. The supernatant above the cell monolayer was removed, and leukotrienes were extracted and analyzed by reversed phase HPLC as described in Materials and Methods. Leukotriene products identified by elution position and ultraviolet spectra were 6-*trans*-$LTB_4$, 6-*trans*-12-*epi*-$LTB_4$ and $LTB_4$. $PGB_2$ is the internal standard.

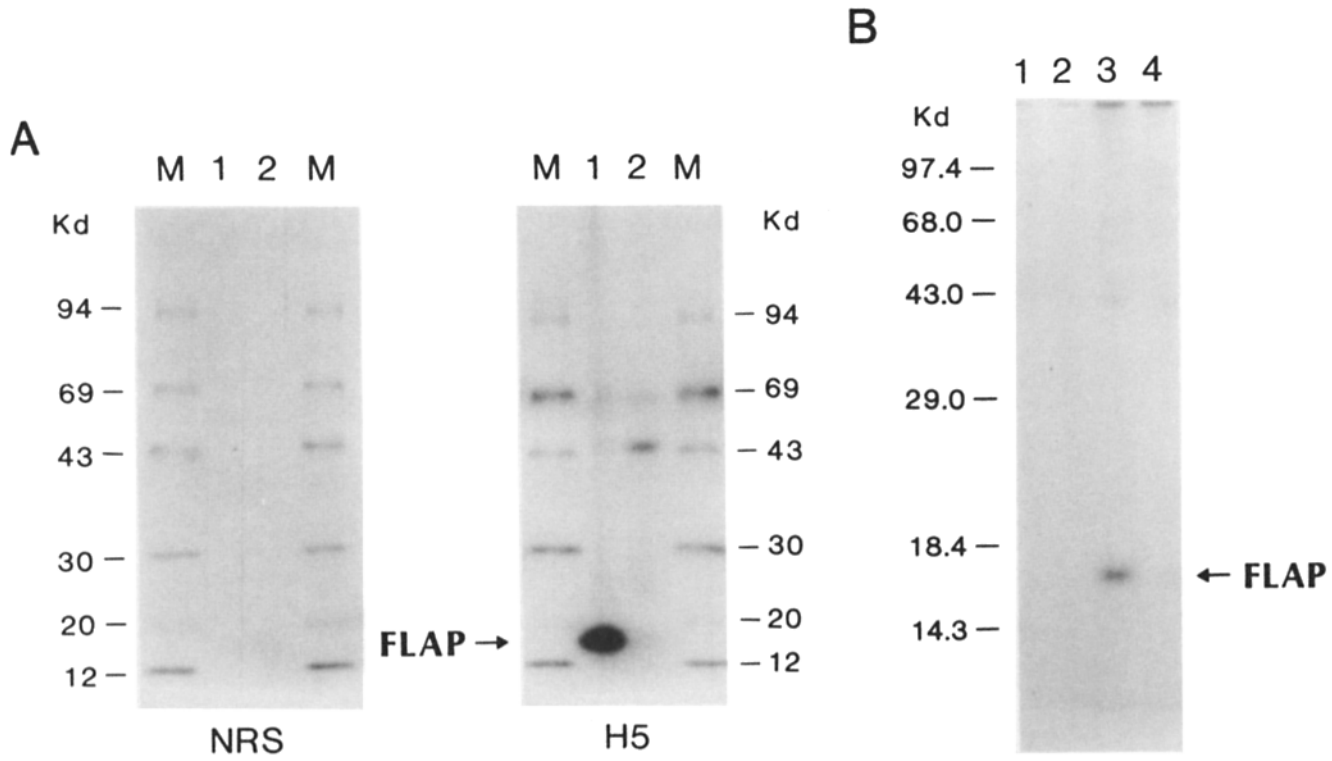


*Figure 2.* Immunoblot analysis and photoaffinity labeling of FLAP in osteosarcoma cells. Lane *A*, immunoblot analysis of FLAP in cells expressing 5-LO only, or cells expressing both 5-LO and FLAP. 100,000 *g* pellet samples (prepared as described in Materials and Methods) were combined with SDS sample buffer, and subjected to electrophoresis and immunoblot analysis as described in Materials and Methods. Blots were exposed to XAR film for ~24 h. Lane *M*, $^{14}$C-labeled molecular weight markers (Amersham Corp.). Lane *1*, samples from osteosarcoma cells expressing 5-LO and FLAP. Lane *2*, samples from osteosarcoma cells expressing 5-LO only. NRS immunoblot was incubated with 1:200 dilution of prebleed for H5. H5 immunoblot was incubated with 1:200 dilution of H5. Lane *B*, photoaffinity labeling and immunoprecipitation of FLAP in cells expressing 5-LO only or 5-LO and FLAP, as described in Materials and Methods. Lanes *1* and *2*, samples from osteosarcoma cells expressing 5-LO only. Lanes *3* and *4*, samples from osteosarcoma cells expressing 5-LO and FLAP. Lanes *1* and *3*, labeling in the absence of 1-μM MK-886. Lanes *2* and *4*, labeling in the presence of MK-886.

challenged and A23187-challenged rat PMN and U937 cells and found no change in membrane localization after A23187 localization (Kargman and Reid, unpublished observations). Competitive photoaffinity labeling followed by immunoprecipitation of membrane-bound FLAP confirms that osteosarcoma cells expressing 5-LO alone have no detectable FLAP and that FLAP expressed in these cells specifically binds leukotriene synthesis inhibitors (Fig. 2 *B*).

### *Translocation of 5-LO in Osteosarcoma Cells Expressing 5-LO and FLAP*

Previous studies have shown that 5-LO translocates to membranes in A23187-stimulated human leukocytes, RBL-1 cells, rat PMN, $Me_2SO$-differentiated HL-60 cells; in fMLP-stimulated $Me_2SO$-differentiated HL-60 cells; and in thapsigargin-, ionomycin- or antigen-stimulated RBL-2H3 cells (16–22, 29). Because FLAP had been shown to be necessary for leukotriene production, we questioned whether this protein is also necessary for the translocation of 5-LO. Osteosarcoma cells expressing both 5-LO and FLAP were preincubated in the absence or presence of 1 μM MK-886 and then stimulated with 1 μM A23187, or $Me_2SO$, as control. After the 10-min incubation, the overlaying medium was removed, extracted and analyzed by HPLC for leukotriene production, the cell monolayer was scraped and 100,000 *g* supernatants and pellets were prepared and analyzed for 5-LO enzyme activity (5-HETE) and 5-LO protein (immunoblot analysis). A typical example of one of the immunoblots of 100,000 *g* supernatants and pellets in osteosarcoma cells expressing both 5-LO and FLAP can be seen in Fig. 3. The results of the leukotriene synthesis and 5-LO translocation and cytosol to membrane fractions in these experiments were quantitated and are expressed in Fig. 4. We were unable to detect leukotrienes from unstimulated cells. Cells stimulated with A23187 synthesized and secreted enzymatic and nonenzymatic isomers of $LTB_4$. Leukotriene synthesis in these cells was inhibited when cells were preincubated in the presence of MK-886 but not when they were preincubated with L-583,916 (Fig. 4 *A*). Fig. 4 *B* demonstrates the A23187-stimulation of cells expressing both 5-LO and FLAP resulted in the loss of ~40% of 5-LO enzyme activity from the supernatant, consistent with that seen previously in human leukocytes, and $Me_2SO$ differentiated HL-60 cells (16–18). We were unable to detect any 5-LO enzyme activity in 100,000 *g* pellets (data not shown). Preincubation of cells with MK-886 resulted in ~90% of the 5-LO enzyme activity being maintained in the supernatant. This was in direct contrast with preincubation of cells with L-583,916, which did not lead to any recovery of the cytosolic 5-LO enzyme activity (Fig. 4 *B*). A similar profile of loss and recovery of immunoreactive 5-LO protein can be seen in Fig. 4 *C*. ~50% of immunoreactive 5-LO protein is lost from the supernatant of A23187-stimulated cells, 70% of which was maintained in

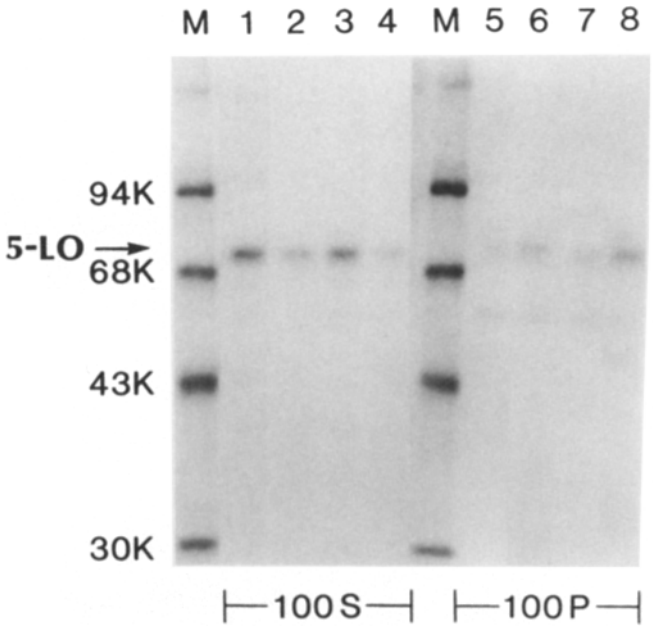


*Figure 3.* A representative immunoblot analysis of 5-LO translocation in osteosarcoma cells expressing 5-LO and FLAP. Samples of 100,000 *g* supernatants (100S) and 100,000 *g* pellets (100P) were combined with volume SDS sample buffer, subjected to electrophoresis and transferred to nitrocellulose membranes as described in Materials and Methods. Blocked membranes were incubated with LO32, a rabbit antibody raised to human leukocyte 5-LO followed by incubation with [$^{125}$I]-protein A (low specific activity). Washed membranes were exposed to XAR film for ~24 h. Lane *M*, molecular weight markers; lanes *1–4*, 100S samples; lanes *5–8*, 100P samples; lanes *1* and *5*, samples from control, untreated cells; lanes *2* and *6*, samples from A23187-treated cells; lanes *3* and *7*, samples from 1-$\mu$M MK-886–preincubated, A23187-treated cells; lanes *4* and *8*, samples from 1-$\mu$M L-583,916–preincubated, A23187-treated cells.

the supernatant when cells were preincubated with MK-886. In agreement with the activity analyses, L-583,916 was unable to prevent the loss of any immunoreactive 5-LO protein from the supernatant after ionophore challenge. Fig. 4 *D* demonstrates the approximate twofold increase in immunoreactive 5-LO protein in the pellet of A23187-stimulated cells. There was almost complete inhibition of the increase of immunoreactive membrane 5-LO protein in A23187-stimulated cells when cells were preincubated in the presence of MK-886. No inhibition of the increase of immunoreactive membrane 5-LO was seen when cells were preincubated in the presence of L-583,916.

### *Translocation of 5-LO in Osteosarcoma Cells Expressing 5-LO and Not Expressing FLAP*

To determine whether FLAP was essential for 5-LO translocation, we performed translocation experiments in osteosarcoma cells expressing 5-LO and not expressing FLAP. Our initial hypothesis was that if FLAP were essential for translocation, cells lacking the protein would be unable to support 5-LO translocation. Experiments identical to those performed with osteosarcoma cells expressing both 5-LO and FLAP were performed. A representative example of an immunoblot analysis from osteosarcoma cells expressing 5-LO but not FLAP is shown in Fig. 5. In agreement with previous findings, we were unable to detect any leukotrienes in cells transfected with 5-LO but not FLAP after stimulation with A23187 (Fig. 6 *A*). Surprisingly, the osteosarcoma cells expressing 5-LO alone showed a 40% loss of 5-LO enzyme activity from the supernatant of A23187-treated cells. As MK-886 specifically interacts with FLAP, this compound did not inhibit 5-LO translocation in this system (Fig. 6 *B*). We were unable to detect significant levels of 5-LO enzyme activity in pellets of A23187-stimulated cells (data not shown). Consistent with the 5-LO activity measurements, there was an ~40% loss of the immunoreactive 5-LO protein from the supernatant of A23187-stimulated cells, and preincubation of cells with either MK-886 or L-583,916 was ineffective (Fig. 6 *C*). Finally, Fig. 6 *D* demonstrates the twofold increase in immunoreactive 5-LO protein in pellets of A23187-stimulated cells, and the lack of inhibition of this increase by either MK-886 or L-583,916.

## *Discussion*

We have used human osteosarcoma cells transfected and expressing either 5-LO only or 5-LO as well as FLAP to examine the mechanism of A23187-induced cellular membrane association and activation of 5-LO. Wild-type osteosarcoma cells express neither 5-LO nor FLAP. In cells expressing both 5-LO and FLAP, we have shown that in response to A23187 stimulation, 5-LO translocates to membranes and leukotrienes are synthesized. A leukotriene synthesis inhibitor (MK-886) that binds selectively to FLAP inhibits 5-LO translocation in these cells. In cells not expressing FLAP, we demonstrated that in response to A23187 stimulation, 5-LO also associates with membranes although in this case leukotrienes are not synthesized. In contrast to osteosarcoma cells expressing both 5-LO and FLAP, MK-886 has no effect on the 5-LO membrane association in cells not expressing FLAP.

These results suggest that A23187-induced 5-LO membrane association and activation can be separated into at least two distinct steps: (*1*) movement to the membrane without product formation and (*2*) activation resulting in product formation. This two-step process is consistent with a recent report by Coffey et al. who have shown membrane association and activation of 5-LO as two distinct and separable events in the rat alveolar macrophage (19). They demonstrated that in the resting rat alveolar macrophage, a large proportion of the 5-LO enzyme activity and immune recognizable protein resides in the membrane fraction. Stimulation of rat alveolar macrophage cells with A23187 does not result in a detectable increase in 5-LO activity or protein in the membrane fraction, yet leukotrienes are synthesized. MK-886 inhibits this A23187-induced leukotriene production. In addition, the 5-LO associated with membranes in the resting rat alveolar macrophage is not dissociated by treatment of cells with MK-886 (19). This is in striking contrast to what has been seen in human PMN, HL-60 cells, and RBL-2H3 cells, where A23187 stimulation causes a significant shift in the distribution of 5-LO from the cytosol to a membrane compartment (16–21, 29). In human leukocytes, translocation of 5-LO is not only inhibited by MK-886 but MK-886 can also reverse 5-LO membrane association after ionophore challenge (22). This suggests that FLAP binding compound has a direct effect on the interaction of 5-LO at a membrane site rather than an indirect effect before 5-LO translocation. Studies on

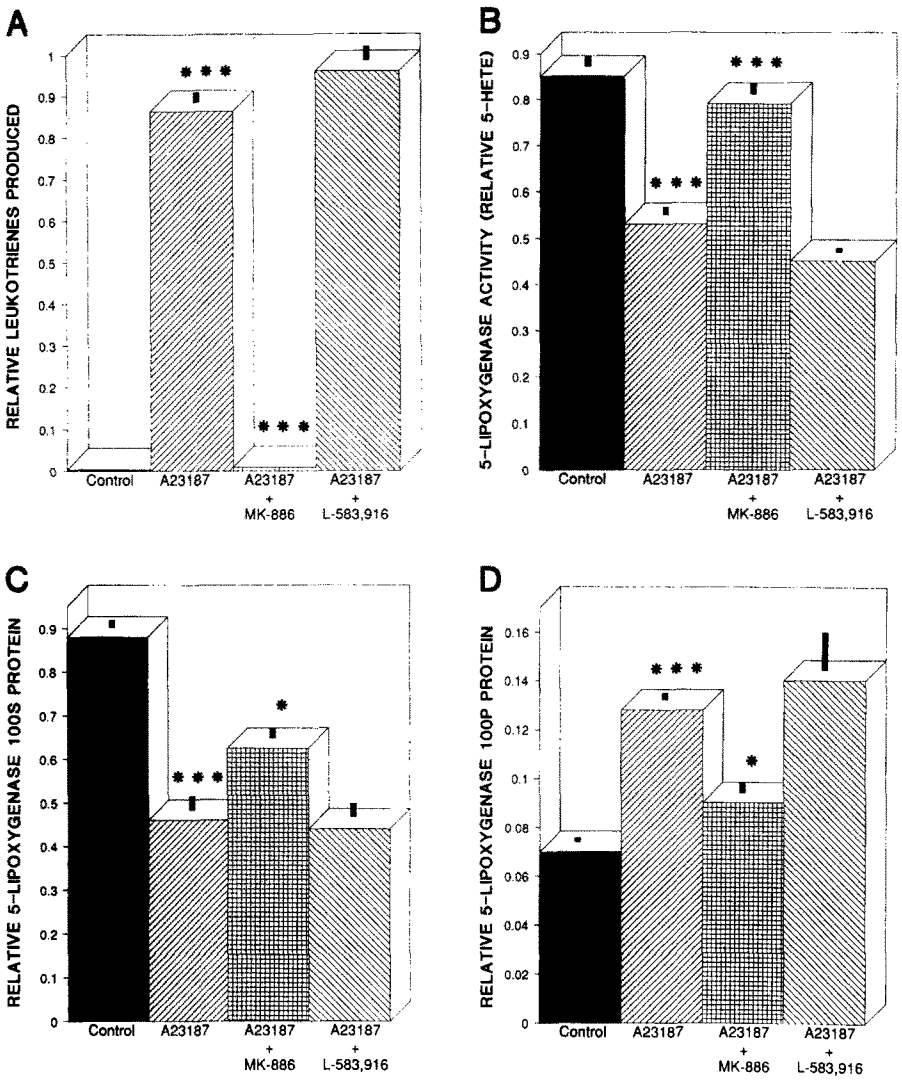


*Figure 4.* Translocation of 5-LO in A23187-stimulated osteosarcoma cells expressing 5-LO and FLAP. Lane *A*, leukotriene synthesis. Lane *B*, 5-LO enzyme activity in 100,000 *g* supernatants (100S). Lane *C*, immunoblot analysis of 5-LO protein in 100,000 *g* supernatants. Lane *D*, immunoblot analysis of 5-LO protein in 100,000 *g* pellets (100P). Cell monolayers were preincubated in the presence or absence of 1 μM MK-886 or L-583,916, followed by incubation in the presence of 1 μM A23187. The supernatants above the monolayer were removed, extracted, and analyzed for leukotrienes by reversed phase HPLC as described in Materials and Methods. 100S samples were assayed for 5-LO enzyme activity (*B*). 100S (*C*) and 100P (*D*) samples were subjected to immunoblot analysis for 5-LO protein. The relative amounts of 5-LO protein were determined by densitometry. The optical density reading from the scanned autoradiograph is expressed as relative 5-LO protein. The most intense 100S and 100P signals were arbitrarily set to 1.0 and the 100P values were corrected for fivefold concentration during resuspension. The values therefore represent the relative concentration of 100S and 100P 5-LO in the cells. Values for leukotriene production, 5-LO activity (5-HETE synthesis), and 5-LO protein represent the mean ±SE for three separate experiments, each performed in duplicate. For immunoblot analyses each sample was loaded in duplicate. At least nine lanes were scanned and their densities averaged for each condition, i.e., control, A23187, or A23187 and MK-886. $^{14}$C-labeled molecular weight marker proteins were loaded on each side of samples to monitor equal transfer of proteins to the nitrocellulose. The differences in leukotriene production, 5-LO enzyme activity (5-HETE) and 5-LO protein between unstimulated and A23187-stimulated cells, and between A23187 challenge in the absence and presence of MK-886 were statistically significant as determined by a *t* test ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$).

MK-886 inhibition of leukotriene synthesis in human phagocytes stimulated by A23187, PAF fMLP, C5a, or $LTB_4$ support this suggestion (30, 31).

The ability of 5-LO to translocate to membranes in response to A23187 stimulation in cells lacking FLAP was surprising. The observation that preincubation of cells with MK-886 had no effect on this membrane association is consistent with our previous data demonstrating that FLAP is the sole selective target of MK-886. Previously, Rouzer and Kargman hypothesized translocated 5-LO was inactive due to enzyme suicide concomitant with leukotriene synthesis (16). When we observed 5-LO associated with membranes in osteosarcoma cells expressing 5-LO alone, we initially predicted that the pelleted enzyme might be active because no product was made. However, 5-LO was inactive in membranes from A23187-stimulated osteosarcoma cells expressing 5-LO alone. Wong et al. have demonstrated that cytosolic 5-LO is rapidly inactivated by incubation with calcium, ATP and arachidonic acid (29). Also in vitro studies with purified recombinant human leukocyte 5-LO have shown the enzyme to inactivate in the presence of calcium, membrane and oxygen (D. Percival, unpublished data). The combination of all three components is necessary for inactivation (D. Percival, unpublished data). Therefore, membrane-associated 5-LO in A23187-challenged osteosarcoma cells could be inactive due to simultaneous exposure to calcium, membrane and arachidonic acid.

Our results with osteosarcoma cells suggest that at least the initial membrane association of 5-LO after A23187 stimulation is the result of an increase in intracellular calcium and can be FLAP independent. Wong et al. demonstrate that in the broken RBL-1 cell (no stimulation), 5-LO association with membranes was calcium concentration dependent over the range of 0.05–5 μM (29). They later showed that stimulation of RBL-2H3 cells with optimal concentrations of ionomycin or thapsigargin triggered comparable increases in intracellular calcium. Only at intracellular calcium concentrations greater than ~300–400 nM did 5-LO associate with membranes and were leukotriene synthesized (20). We have recently shown that HL-60 cell 5-LO translocates to the

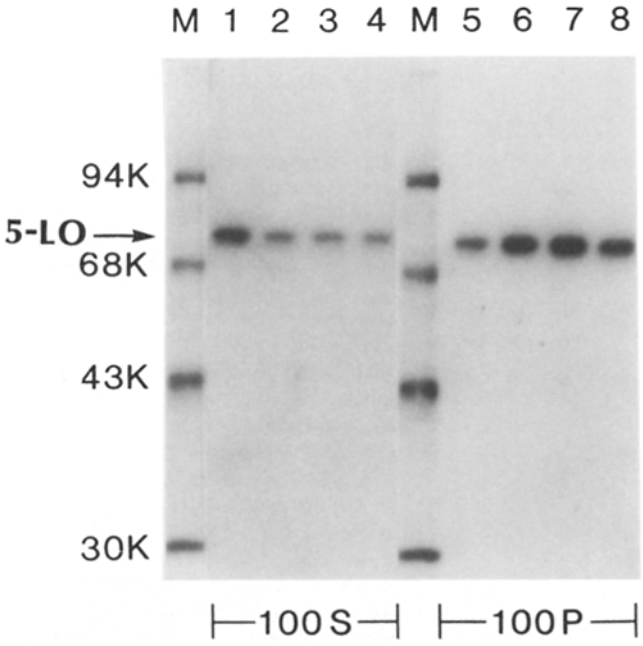


*Figure 5.* A representative immunoblot analysis of 5-LO protein in osteosarcoma cells expressing 5-LO only. 100S and 100P samples were prepared and analyzed as described in the legend for Fig. 3. The identities of the lanes are as follows: lane *M*, molecular weight markers; lanes *1–4*, 100S samples; lanes *5–8*, 100P samples; lanes *1* and *5*, samples from unstimulated cells; lanes *2* and *6*, samples from A23187-stimulated cells; lanes *3* and *7*, samples from A23187-stimulated cells; preincubated with MK-886 and lanes *4* and *8*, samples from A23187-stimulated cells preincubated with L-583,916.

membrane in response to the receptor-mediated stimulus, fMLP (18). Although we did not measure the change in intracellular calcium after stimulation of cells with fMLP, reported studies indicate a maximum intracellular calcium concentration of ~300 nM in fMLP-stimulated cells (C. Chan, unpublished data). The low amount of leukotriene production, 5-LO translocation and change in intracellular calcium after stimulation of cells with fMLP correlates well with results of Wong et al. who show that ~15% of rat RBL-2H3 cell 5-LO associated with membranes at intracellular calcium concentrations such as those that would be elicited by fMLP stimulation (20). Wong et al. also demonstrated that removal of calcium from the medium before stimulation of rat RBL-2H3 cells with ionomycin or thapsigargin eliminated 5-LO translocation and leukotriene synthesis, again implying 5-LO membrane association is calcium dependent (20). Wong et al. have recently shown that antigen stimulation induced ~15% translocation of 5-LO and this translocation was inhibited by MK-886 (27). This antigen-induced 5-LO-translocation had both a calcium-dependent and calcium-independent component (21).

Proteins other than 5-LO have been shown to associate with membrane in a calcium-dependent fashion. At intracellular calcium concentrations such as those seen in activated cells (~300 nM), the arachidonoyl-hydrolyzing cytosolic phospholipase $A_2$ ($cPLA_2$) translocates in vesicular systems to membranes (32, 33). Furthermore, a 140–amino acid fragment of $cPLA_2$ containing a region homologous to the constant region 2 of protein kinase C (PKC C-2) translocates to membranes in a calcium-dependent manner (33). Several platelet PKC isoforms have been shown to translocate to membranes in the presence of 1 $\mu$M PMA (34). Other proteins with C-2–like domains have been found to associate with phospholipids, among which are synaptic vesicle protein p65 and GTPase activating protein (GAP) (35, 36). 5-LO does not contain a C-2–like PKC domain (9). Therefore, the membrane association of 5-LO appears to differ from that of $cPLA_2$ and other C-2–containing proteins.

It is important to note that although our results with osteosarcoma cells suggest that 5-LO membrane association can occur in the absence of FLAP, we are unaware of any normal cell type in which 5-LO exists in the absence of FLAP. In all cells (except alveolar macrophage) in which 5-LO translocation has been investigated (human and rat PMN, RBL-1, RBL-2H3, and HL-60 cells) MK-886 has inhibited both translocation of 5-LO and leukotriene synthesis. Therefore, 5-LO membrane movement may in the majority of cell types expressing 5-LO be modulated by the presence of both calcium and FLAP. It is clear that the rat alveolar macrophage is an exception to this rule and the initial association of 5-LO to membranes in this cell type appears to be FLAP independent (19). Nevertheless, Coffey et al. demonstrate that leukotriene synthesis is inhibited by MK-886 in the A23187-stimulated rat alveolar macrophage, consistent with our results that leukotriene synthesis is dependent on an interaction between 5-LO and FLAP (19).

As a result of our studies, we propose a number of models to explain 5-LO translocation to membranes in intact cells in the presence or absence of FLAP. These models also visualize how MK-886 binding to FLAP may prevent 5-LO membrane association. The models are depicted in Fig. 7. A 5-LO membrane binding site (MBS) may be comprised of a number of proteins involved in the leukotriene synthetic pathway. The membrane binding site may be exclusively lipid. In cells lacking FLAP (Fig. 7 *A*), an increase in intracellular calcium after A23187 activation facilitates the association of 5-LO with the MBS. Calcium may alter the conformation or aggregation state of 5-LO itself and thereby facilitate membrane association. Because FLAP is essential for leukotriene synthesis leukotrienes are not synthesized in the absence of FLAP expression (Fig. 7 *A*). Preincubation of these cells in the presence of MK-886 has no effect on the membrane association of 5-LO as MK-886 specifically binds to FLAP. Hence, 5-LO translocation can be considered calcium dependent and FLAP independent. In cells expressing FLAP (Fig. 7 *B*), an increase in intracellular calcium after A23187 activation also facilitates the association of 5-LO with the membrane. However, in this case, leukotrienes are synthesized because FLAP is present in these cells. The exact way in which FLAP activates leukotriene synthesis is unclear but it is possible FLAP facilitates the transfer of arachidonic acid or 5-HPETE to 5-LO. In whole cells, in contrast to broken cell assays, production of leukotrienes relative to 5-Hete is enhanced, so FLAP may alter the conformation of 5-LO to facilitate the concerted oxidation and dehydration reactions. Fig. 7 *C* portrays a cell containing FLAP and preincubated in the presence of MK-886. In Fig. 7 *C*, the membrane binding site at which 5-LO interacts has undergone a conformational change after binding of MK-886 to FLAP. 5-LO is unable to interact with the altered membrane site; it remains in the cytosol and is unable to interact with its substrate arachidonic acid. Consequently, leukotrienes

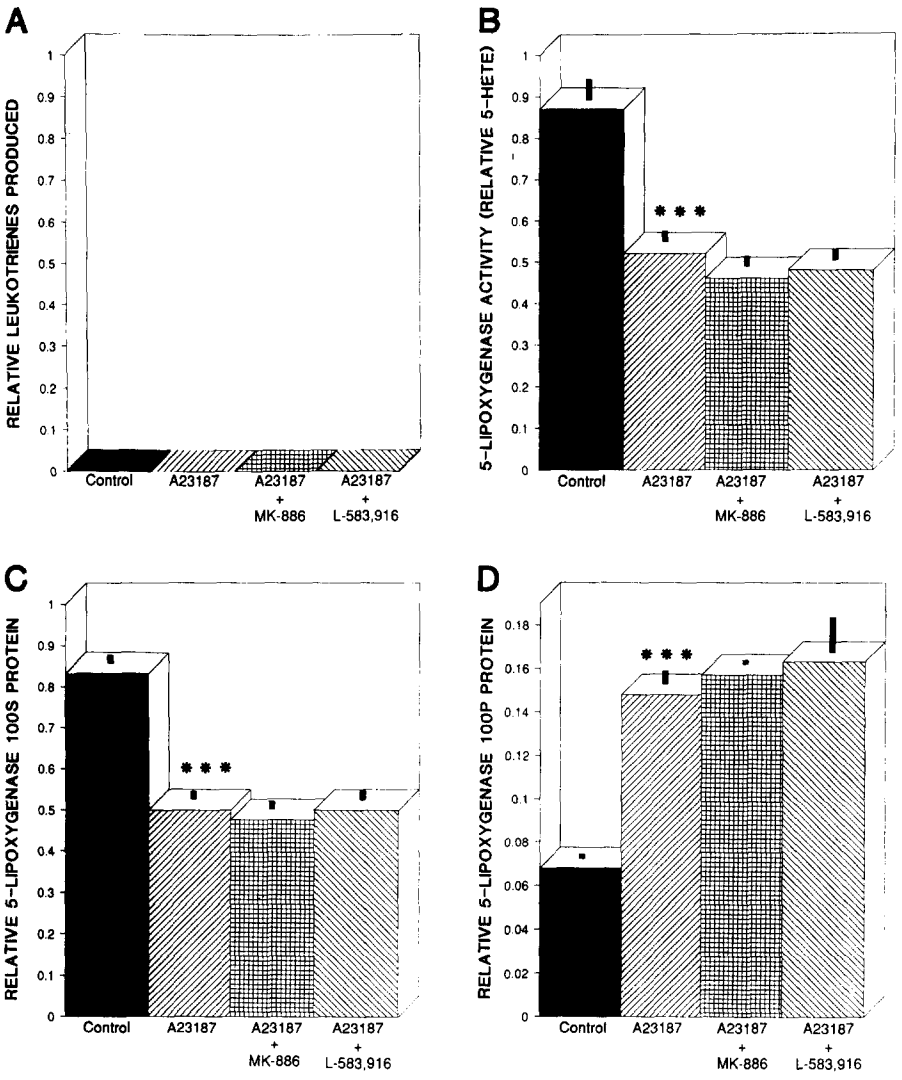


*Figure 6.* Translocation of 5-LO in A23187-stimulated osteosarcoma cells expressing 5-LO only. Lane *A*, leukotriene synthesis. Lane *B*, 5-LO enzyme activity in 100,000 *g* supernatants. Lane *C*, immunoblot of 5-LO protein in 100,000 *g* supernatants. Lane *D*, immunoblot analysis of 5-LO protein in 100,000 *g* pellets. Cell monolayers were preincubated in the presence or absence of 1 $\mu$M MK-886 or 1 $\mu$M L-583,916 and then incubated in the presence or absence of 1 $\mu$M A23187. The supernatant above the monolayer was removed, extracted and leukotriene production (*A*) analyzed by reversed phase HPLC as described in Materials and Methods. 100S samples were analyzed for 5-LO enzyme activity (*B*) and 5-LO protein by immunoblot analysis (*C*). 100P samples were also analyzed for 5-LO protein by immunoblot analysis (*D*). The relative amounts of 5-LO protein were determined by densitometry and are expressed as described in the legend for Fig. 4. Values for leukotriene production, 5-LO enzyme activity and 5-LO protein represent the mean ±SE for two separate experiments each performed in duplicate. For immunoblot analyses, each sample was loaded in duplicate. At least six lanes were scanned and their densities averaged for each condition, i.e., control, A23187, or A23187 and MK-886. $^{14}$C-labeled molecular weight marker proteins were loaded on each side of samples to monitor equal transfer of proteins to the nitrocellulose. The differences in leukotriene production, 5-LO enzyme activity and 5-LO protein between unstimulated and A23187-stimulated samples were statistically significant as determined by a *t* test (***$P < 0.002$).

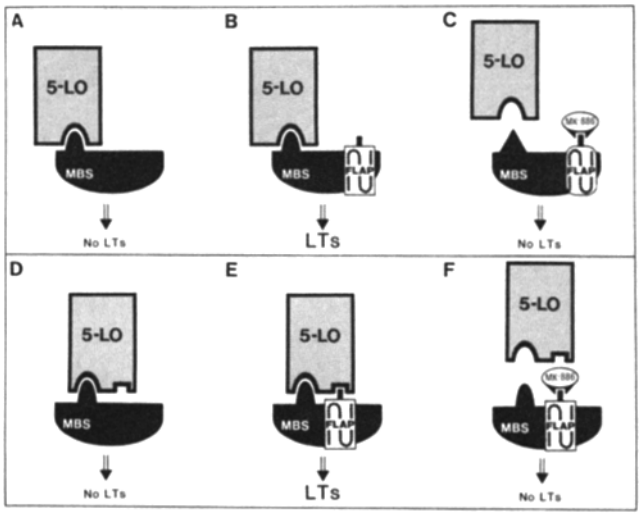


*Figure 7.* Models for A23187-induced translocation of 5-LO. A hypothetical membrane binding site (*MBS*) may be a multienzyme complex containing proteins in the leukotriene synthetic pathway or may be lipid only. Lane *a*, upon A23187 stimulation, a rise in intracellular calcium results in the membrane association of 5-LO. This may be due to a conformational change in 5-LO or a change in 5-LO aggregation state. Because FLAP is absent, leukotrienes are not synthesized. Lane *B*, upon A23187 stimulation, a rise in intracellular calcium results in the membrane association of 5-LO; "activation" of 5-LO by FLAP results in the formation of leukotrienes. Lane *C*, preincubation of cells expressing FLAP with MK-886 leads to high affinity binding of MK-886 to FLAP; this results in a conformational change in the membrane binding site at which 5-LO interacts. This alteration in 5-LO binding site may be induced by a conformational change in FLAP itself. After A23187 stimulation, despite increased calcium concentrations, 5-LO is unable to interact with its membrane binding site and the synthesis of leukotrienes is effectively inhibited. Lanes *D–F* portray a model in which there is a direct interaction between 5-LO and FLAP. Lane *D*, after A23187 stimulation 5-LO binds to the MBS. Lane *E*, after A23187 stimulation, a direct interaction of 5-LO and FLAP occurs at the MBS resulting in the formation of leukotrienes. Lane *F*, MK-886 binds to FLAP and prevents 5-LO membrane association via steric inhibition blocking a direct protein-protein interaction of 5-LO and FLAP.

are not synthesized. This model implies that a conformational change resulting from binding of MK-886 to FLAP may prevent 5-LO membrane association. The conformational change in the membrane binding site for 5-LO may be induced by a conformational change in FLAP itself. This model does not require a direct association of 5-LO and FLAP. The model shown in Fig. 7 *D–F* predicts a direct protein-protein association of 5-LO and FLAP. Binding of MK-886 to FLAP may prevent 5-LO membrane association by way of steric inhibition as depicted in Fig. 7 *F*. Because MK-886 is relatively small in comparison to 5-LO and FLAP, steric inhibition would be more probable if 5-LO and FLAP directly interact. However, to date we have not been able to show direct interaction of 5-LO and FLAP (S. Kargman, P. Vickers, and D. K. Miller, unpublished data). *D–F* shows 5-LO binding at two sites, one of which can be a direct interaction with FLAP. Whether 5-LO binds directly to FLAP and if so if it is the same site at which MK-886 binds is unclear. We are currently investigating these possibilities by site-specific and deletion mutagenesis analysis of FLAP in several recombinant expression systems. Both models predict 5-LO association at the same cellular membrane as FLAP. We are currently addressing this prediction by electron micrographic immunohistochemical localization studies on 5-LO and FLAP.

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