Correlation between Expression of 5-Lipoxygenase-activating Protein, 5-Lipoxygenase, and Cellular Leukotriene Synthesis*

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Previous studies involving transfection of cDNAs for 5-lipoxygenase-activating protein (FLAP) and 5-lipoxygenase into osteosarcoma cells have shown that both these proteins are essential for leukotriene synthesis (Dixon, R. A. F., Diehl, R. E., Opas, E., Rands, E., Vickers, P. J., Evans, J. F., Gillard, J. W., and Miller, D. K. (1990) Nature 343, 282-284). In the present study we show that FLAP is present in a variety of cells known to produce leukotrienes, but is absent from a number of cells which do not synthesize leukotrienes. Furthermore, differentiation of the human promyelocytic HL-60 cell line towards granulocytic cells following exposure to dimethylsulfoxide is associated with the concurrent induction of both FLAP and 5-lipoxygenase and an increased capacity to synthesize leukotrienes. Cellular leukotriene synthesis in this system is functionally dependent on FLAP as shown by its inhibition by the leukotriene biosynthesis inhibitor MK-886, a compound which specifically binds to FLAP.

Leukotrienes are arachidonic acid metabolites formed by leukocytes and other myeloid cells following immunologic and inflammatory stimuli (1-4). Due to the biological properties of leukotrienes, which include enhancement of leukocyte chemotaxis and chemokinesis, production of vascular permeability changes, pulmonary constriction, mucous hypersecretion, vasoconstriction, and modulation of a number of immune responses, considerable effort over the past decade has been directed toward the development of inhibitors of leukotriene formation (5-8). Most inhibitors have been targeted against 5-lipoxygenase, the first enzyme in the leukotriene biosynthetic pathway, which catalyses both the oxygenation of arachidonic acid to 5-HPETE and the subsequent dehydration of 5-HPETE to the unstable epoxide LTA₄. Most of the known 5-lipoxygenase inhibitors appear to function through a redox mechanism potentially involving the generation of radical species which may be associated with drug toxicity (8-10). Recently, a novel class of indole leukotriene biosynthesis inhibitors has been described which do not act directly on 5-lipoxygenase but rather prevent its membrane association and cellular activation (11, 12). This class is exemplified by MK-886, a compound which inhibits leukotriene biosynthesis in all inflammatory cells so far examined (11-14). The specific protein target of MK-886 has been identified in neutrophils as a membrane-bound 18-kilodalton protein termed 5-lipoxygenase-activating protein (FLAP) (15). Osteoarcoa cells cotransfected with FLAP and 5-lipoxygenase synthesized leukotrienes in response to ionophore challenge in contrast to cells transfected with FLAP or 5-lipoxygenase alone (16). Therefore, both FLAP and 5-lipoxygenase are necessary for cellular leukotriene synthesis.

In the present study we have investigated the occurrence of FLAP by immunoblot analysis in a variety of cells and have shown a positive relationship between the expression of FLAP and the ability of cells to produce leukotrienes. The relationship between FLAP, 5-lipoxygenase, and leukotriene synthesis has also been investigated during cellular differentiation of the human promyelocytic cell line HL-60 (17). The HL-60 cell line has been extensively characterized for its ability to differentiate towards different myeloid cell types in response to specific stimuli (18, 19). In response to MeSO₄, HL-60 cells acquire morphological and functional characteristics of mature neutrophils, including an increase in 5-lipoxygenase mRNA (20), an increased capacity to synthesize leukotrienes (21, 22) and an appearance of specific LTβ₁ binding sites (23). We demonstrate here that the increased leukotriene synthetic activity of MeSO₄ differentiated HL-60 cells is associated with the induction of both FLAP and 5-lipoxygenase.

We show that leukotriene synthesis in the MeSO₄ differentiated HL-60 cells is abolished by preincubation of the cells with MK-886, a compound that specifically binds to FLAP.

MATERIALS AND METHODS

Cell Culture—HL-60 cells, originally obtained from the American Type Culture Collection, were grown at 37 °C in Iscove's modified Dulbecco's medium, supplemented with 20% heat-inactivated fetal bovine serum (Sigma), 50 units/ml penicillin, 50 µg/ml streptomycin, thylpropanoic acid); FLAP, 5-lipoxygenase-activating protein; MeSO₄, dimethyl sulfoxide; 100S, 100,000 × g supernatant; 100P, 100,000 × g pellet; 3[125I]-L-669,063, 3-[1-(4-hydroxy-3-iodophenyl)-methyl]-3-(4-azidophenylsulfonyl)-5-isopropylindol-2-yl]-2,2-dime-thylthromboxanic acid, where iodo is the radionuclide form of 125Iiodine.
and 2 mM L-glutamine (Flow Laboratories, Inc., McLean, VA) under an atmosphere of 6% CO2. To induce myeloid differentiation, cells were subcultured to 0.2 x 10^6 cells/ml and exposed to 1.3% MeSO (Sigma) for up to 5 days. Morphological differentiation of cells was confirmed by staining with May-Grünewald-Giemsa stain. After exposure to MeSO for 5 days, approximately 85% of cells morphologically appeared to be mature granulocytes. Control cultures showed approximately 10% spontaneously differentiated cells, based on this morphological staining.

**Cell Lysis and Subcellular Fractionation**—Frozen cell pellets were thawed on ice in homogenization buffer (50 mM potassium phosphate, 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) at a density of 6.6 x 10^6 cells/ml and sonicated at 4°C by three 20-s bursts using a Cole-Parmer Instrument Co. (Chicago, IL) 4710 series ultrasonic homogenizer set at 75% duty cycle and power level 3. Sonicated cells were centrifuged at 10,000 x g for 15 min at 4°C, and the resulting supernatant was recentrifuged at 100,000 x g for 60 min at 4°C. The 100,000 x g supernatants (100s) were collected and the pellets (100P) rinsed with homogenization buffer and then resuspended in fresh homogenization buffer (1/5 volume of the original supernatant) using a Potter-Elvehjem homogenizer. Samples were analyzed for 5-lipoxygenase activity (100S) or [131I]-L-669,083 labeling (100P) or processed for immunoblot analysis (100S and 100P) as described below.

**SDS-PAGE and Immunoblot Analysis of 5-Lipoxygenase and FLAP**—100S and 100P samples were mixed with 1/5 volume of SDS-PAGE sample buffer (20 mM Tris-HCl, pH 6.5, 0.5% SDS, 4% glycerol, 0.04% 2-mercaptoethanol, 0.1 mg/ml bromophenol blue) and stored immediately for 5 min to prevent proteolysis (24). Proteins were then separated by SDS-PAGE on 10, 12.5, or 10–20% polyacrylamide gels by the method of Laemmli (25). Immunoblots for 5-lipoxygenase (100S) and FLAP (100P) were performed after transferring proteins from the gels to nitrocellulose (26) or to PVDF membranes (15). Nonspecific sites on the nitrocellulose were blocked with 3% bovine serum albumin in 1% gelatin/TTBS. Blots were washed twice with TTBS and incubated with 5 x 10^6 cpm/μl [131I]-protein A (low specific activity, Du Pont-New England Nuclear) in 1% gelatin/TTBS for 90 min to room temperature. Blots were washed five times with 50 mM phosphate buffer, pH 7.1, 0.1 M NaCl (NTTBS) and 1% Triton X-100, 0.5% Nonidet P-40, and 10,000 x g for 15 min at 4°C, and the resulting supernatant was recentrifuged at 100,000 x g for 60 min at 4°C. The 100,000 x g supernatants (100S) were collected and the pellets (100P) rinsed with homogenization buffer and then resuspended in fresh homogenization buffer (1/5 volume of the original supernatant) using a Potter-Elvehjem homogenizer. Samples were analyzed for 5-lipoxygenase activity (100S) or [131I]-L-669,083 labeling (100P) or processed for immunoblot analysis (100S and 100P) as described below.

**Correlation between Expression of FLAP, 5-Lipoxygenase, and Cellular Leukotriene Synthesis**

**SDS-PAGE and Immunoblot Analysis of 5-Lipoxygenase and FLAP**—100S and 100P samples were mixed with 1/5 volume of SDS-PAGE sample buffer (20 mM Tris-HCl, pH 6.5, 0.5% SDS, 4% glycerol, 0.04% 2-mercaptoethanol, 0.1 mg/ml bromophenol blue) and stored immediately for 5 min to prevent proteolysis (24). Proteins were then separated by SDS-PAGE on 10, 12.5, or 10–20% polyacrylamide gels by the method of Laemmli (25). Immunoblots for 5-lipoxygenase (100S) and FLAP (100P) were performed after transferring proteins from the gels to nitrocellulose (26) or to PVDF membranes (15). Nonspecific sites on the nitrocellulose were blocked with 3% bovine serum albumin in 1% gelatin/TTBS. Blots were washed twice with TTBS and incubated with 5 x 10^6 cpm/μl [131I]-protein A (low specific activity, Du Pont-New England Nuclear) in 1% gelatin/TTBS for 90 min to room temperature. Blots were washed five times with 50 mM phosphate buffer, pH 7.1, 0.1 M NaCl (NTTBS) and 1% Triton X-100, 0.5% Nonidet P-40, and 10,000 x g for 15 min at 4°C, and the resulting supernatant was recentrifuged at 100,000 x g for 60 min at 4°C. The 100,000 x g supernatants (100S) were collected and the pellets (100P) rinsed with homogenization buffer and then resuspended in fresh homogenization buffer (1/5 volume of the original supernatant) using a Potter-Elvehjem homogenizer. Samples were analyzed for 5-lipoxygenase activity (100S) or [131I]-L-669,083 labeling (100P) or processed for immunoblot analysis (100S and 100P) as described below.

**PMN Monocyte B Cell T Cell**

**Fig. 1. Immunoblot analysis of FLAP in a selection of leukocytes and myeloid and lymphoid cell lines.** Membranes of cells were prepared and mixed with 2 x 50% SDS sample buffer to a final concentration of 1 mg protein/ml as measured by the Pierce Chemical Co. BCA protein assay reagent. Amounts for each cell type loaded onto the 10–20% gradient gels were: rat peritoneal neutrophils (PMN, 5 μg), human platelets (20 μg), human monocytes (20 μg), murine B-cells (20 μg), murine T-cells (20 μg), human THP.1 monocytic cells (20 μg), rat RAW 264.7 macrophage cells (40 μg), murine CXBG Mastc-1 mastocyte cells (20 μg), murine P815 mastocytes (20 μg), murine T-cell lines (20 μg of OKT3, E14, and BW5 cells), and murine YAC B-cells (20 μg). Following electrophoresis, the gels were transferred to PVDF membranes and immunoblotted as described under "Materials and Methods." Rat peritoneal neutrophils were prepared from casein-elicted rats as previously described (15). Normal human platelet membranes were a gift of Dr. S.-B. Hwang. Normal human monocytes and B-cells were purified from leukophoresed blood (University of Pennsylvania Hospital) by elutriation (39). Normal human T-cells were obtained by rosetting mononuclear cells with sheep erythrocytes followed by centrifugation on a ficoll-hypaque gradient and hypotonic lysis to remove the erythrocytes (39). All cell lines were passaged in vitro except CXBG cells which were passaged weekly as described (40).

**Fig. 2. RNA blot analysis of 5-lipoxygenase and FLAP in undifferentiated and MeSO-differentiated HL-60 cells.** RNA was isolated and Northern blot analysis performed as described under "Materials and Methods." 0, 18, 24, and 48 refer to hours of exposure of HL-60 cells to 1.3% MeSO. Migration positions of marker RNA species are indicated on the right. Single RNA species corresponding to 2.7 and 1.0 kilobase for 5-lipoxygenase and FLAP, respectively, were detected. Equal RNA loading per lane was confirmed by ethidium bromide staining of ribosomal RNA (data not shown).
Correlation between Expression of FLAP, 5-Lipoxygenase, and Cellular Leukotriene Synthesis

FIG. 3. Immunoblot analysis of 5-lipoxygenase and FLAP protein in HL-60 cells following exposure to Me₂SO. HL-60 cells were grown in the absence or presence of 1.3% Me₂SO and processed for SDS-PAGE immunoblot analyses as described under "Materials and Methods." The insert shows autoradiograms from 100s samples for 5-lipoxygenase (5-LO) and 100P samples for FLAP where: S, standard references of human leukocyte 5-lipoxygenase or FLAP; 1, control cells at 0.2×10⁶ cells/ml prior to exposure to Me₂SO; 2–6, HL-60 cells exposed to 1.3% Me₂SO for 18, 24, 48, 72, and 96 h; and 7, 96-h control-undifferentiated cells at 1.6×10⁶ cells/ml.

Fig. 4. 5-Lipoxygenase activity in 100s samples from HL-60 cells following exposure to Me₂SO. HL-60 cells were grown in the absence or presence of 1.3% Me₂SO and assayed for 5-lipoxygenase activity as described under "Materials and Methods." 5-Lipoxygenase activity is expressed as nanomoles of 5-HETE produced/mg of protein/10-min incubation as described under "Materials and Methods."

resuspended in SDS-PAGE sample buffer, boiled for 5 min, and separated by SDS-PAGE on 13.5% gels (25). The gels were dried and exposed to XAR-5 film at −70 °C for 48–120 h.

5-Lipoxygenase Assay—5-Lipoxygenase activity was determined in 0.5-ml reaction volumes containing 100 μM arachidonic acid, 2 μM 15-hydoxyprost-11,13-eicosadienoic acid, 0.1 M Tris-HCl, 3 mM CaCl₂, 1.6 mM EDTA, 2 mM ATP, 1 mM dithiothreitol, 2.5% glycerol, 30 mM potassium phosphate, and 100s protein sample (700–1700 pg of protein) at a final pH of 7.5. After incubation for 10 min at 37 °C, 0.5 ml of ethanol containing prostaglandin B₂ (1 nmol/ml) was added, samples mixed, and precipitated protein removed by centrifugation at 12,000 × g for 5 min at 4 °C. Aliquots of the resulting supernatants were analyzed by reversed-phase HPLC as previously described (24). Under these conditions 5-HETE and 5-HPETE are the major 5-lipoxygenase products and 5-lipoxygenase activity is measured as nmol 5-HETE and 5-HPETE produced per mg of protein per 10-min incubation.

Leukotriene Synthesis in HL-60 Cells and Its Inhibition by MK-886—HL-60 cells were harvested by centrifugation, washed once in Dulbecco’s phosphate buffered saline, and resuspended at 2×10⁶ cells/ml in Dulbecco’s phosphate buffer saline prewarmed to 37 °C. MK-886 in Me₂SO or Me₂SO alone was added, and the cells incubated for 5 min at 37 °C. Calcium ionophore A23187 was added from a 1 mM stock in Me₂SO to a final concentration of 1 μM and incubation continued for a further 10 min at 37 °C. The concentration of Me₂SO was constant in all samples and never exceeded 0.4%. A 1-ml cell sample was added to 1 ml of ethanol containing prostaglandin B₂ (1 nmol/ml). Samples were extracted as previously described (24) and analyzed by reversed-phase HPLC using the conditions indicated in the legend to Fig. 6.

RESULTS

A selection of myeloid-derived cell lines were investigated for the presence of FLAP by immunoblot analysis (Fig. 1). Using a peptide antibody to the rat FLAP 1–39 amino acid sequence it had been shown earlier that FLAP was detected in similar amounts in both rat and human leukocyte membranes (15). When membrane fractions of normal human, rat, and murine leukocytes and a number of leukocytic cell lines were investigated for the presence of FLAP, only those cells able to produce leukotrienes contained significant concentrations of FLAP (Fig. 1). Human monocytes known to produce both LTβ and LTC₄ (30–33) contained FLAP but less than 25% of that found in neutrophils. FLAP was not detected in platelets or T-cells, and only a trace amount of FLAP was found in B-cells. In contrast to normal monocytes, FLAP was not detected in the human THP-1 monocytic cell line. The rat macrophage cell line RW2647 had a small amount of FLAP; in contrast to murine C3BGAMet-1 mastocytoma cells which contained substantial amounts of FLAP, FLAP was absent in murine P815 mastocytoma cells, EL4, BW5, and YAC T-lymphocytic cell lines as well as murine B-cell hybridoma OKT3 cells (Fig. 1). The relative concentration of FLAP in these cells correlated well with previous reports of 5-lipoxygenase concentrations and to their known capacity to synthesize leukotrienes (4).

The human promyelocytic HL-60 cell line (17–23) was used to further investigate the expression of FLAP, 5-lipoxygenase, and leukotriene synthesis. In undifferentiated HL-60 cells FLAP mRNA was undetectable while a low concentration of 5-lipoxygenase mRNA was observed (Fig. 2). An increase was shown in both FLAP and 5-lipoxygenase mRNA in cells after exposure to Me₂SO (Fig. 2). These increased concentrations continued for a further 10 min at 37 °C. The concentration of Me₂SO was constant in all samples and never exceeded 0.4%. A 1-ml cell sample was added to 1 ml of ethanol containing prostaglandin B₂ (1 nmol/ml). Samples were extracted as previously described (24) and analyzed by reversed-phase HPLC using the conditions indicated in the legend to Fig. 6.
Correlation between Expression of FLAP, 5-Lipoxygenase, and Cellular Leukotriene Synthesis

FIG. 6. Leukotriene production in HL-60 cells. A, undifferentiated HL-60 cells; B, 5-day MeSO-differentiated HL-60 cells; C, 5-day MeSO-differentiated HL-60 cells incubated with MK-886. Cell suspensions (2 \times 10^7 cells/ml) were incubated for 10 min at 37 °C with 1 μM A23187 in the absence (A and B) or presence (C) of 1 μM MK-886. Leukotrienes were extracted and analyzed by reversed-phase HPLC on a Waters C18 Novapak column eluting with acetonitrile/methanol/water/acetic acid (28:18:54:1) at a flow rate of 1.2 ml/min. Products were monitored at 270 nm and LTc4, LTD4, and LTB4 concentrations calculated by comparison with an internal standard prostaglandin B2 (PGB2) correcting for relative absorption maxima for LTC4, LTD4, LTB4, and PGB2.

FIG. 7. Inhibition of MK-886 of leukotriene synthesis in MeSO-differentiated HL-60 cells. 5-day MeSO-differentiated HL-60 cells (2 \times 10^7 cells/ml) were incubated for 3 min at 37 °C in the presence of various concentrations of MK-886. Calcium ionophore A23187 (1 μM) was added and cells incubated for a further 10 min at 37 °C. Leukotriene production was measured as described in the legend for Fig. 6. LTC4, LTD4, and LTB4 concentrations were pooled for total leukotriene concentration. Results are the means of duplicate determinations with the range of duplicates indicated.

Enzyme activity and photoaffinity labeling studies were carried out to confirm the identities of 5-lipoxygenase and FLAP, respectively. 5-Lipoxygenase activity in cytosolic fractions from HL-60 cells increased for 4 days after exposure of HL-60 cells to MeSO (Fig. 4). Undifferentiated HL-60 cells had 10–15% of the 5-lipoxygenase activity of 4-day MeSO-differentiated HL-60 cells (Fig. 4). The photoaffinity ligand 125I L-669,083 which was used in the initial characterization of FLAP was used to confirm levels of FLAP during HL-60 differentiation. Photoaffinity labeling of HL-60 proteins by 125I-L-669,083 revealed a large number of nonspecifically labeled proteins in both 100S (data not shown) and 100P (Fig. 5) fractions but only the labeling of an 18-kilodalton 100P protein consistently increased 3-4-fold after 5-day exposure to MeSO (Fig. 5A). As shown in Fig. 5A the photoaffinity labeling of this 18-kilodalton protein was selectively inhibited by preincubation with MK-886. When 125I-L-669,083 photoaffinity labeling was followed by immunoprecipitation with antisera raised against a peptide sequence of FLAP, only one radioactive band was immunoprecipitated (Fig. 5D). Detectable amounts of this immunoprecipitated protein increased from 2 to 4 days following exposure of HL-60 cells to MeSO (Fig. 5). The photoaffinity labeling of the immunoprecipitated protein was inhibited by preincubation with MK-886 (data not shown).

Cellular production of leukotrienes following calcium ionophore activation of HL-60 cells was demonstrated in both undifferentiated and 5-day MeSO-differentiated HL-60 cells (Fig. 6). Leukotriene products identified were LTB4, LTD4, and LTA4 (Fig. 6B). After exposure to MeSO for 5 days approximately a 4-fold increase in each leukotriene product was observed with concentrations of 0.18, 0.17, and 0.10 nmol/10^6 cells increasing to 0.63, 0.82, and 0.35 nmol/10^6 cells for LTB4, LTD4, and LTA4, respectively (Fig. 6, A and B). The identities of the leukotrienes were confirmed by coelution with the appropriate reference standards and also by radioimmunoassay for LTC4 and LTD4 (data not shown). Two unidentified peaks were observed in the HPLC profile after

of mRNA are associated with a similar increase in the levels of immunoreactive FLAP and 5-lipoxygenase after exposure to MeSO (Fig. 3). Concentrations of immunoreactive 5-lipoxygenase in the HL-60 cells plateau after 2–4 day exposure to MeSO while concentrations of FLAP decrease slightly after 2 days. Immunoblots of protein from undifferentiated cells were performed at both the initial subculture density of 0.2 \times 10^6 cells/ml and at the density reached after culture for 4 days (1.8 \times 10^6 cells/ml) to control for any possible changes in expression due to cell density. No differences in FLAP or 5-lipoxygenase immunoreactive concentrations were observed at these different cell concentrations.
ionophore challenge of the MeSO-differentiated HL-60 cells (Fig. 6B). These migrate in a position similar to that of the all-trans-nonenzymatic LTB4 isomers but were not definitively identified. Preincubation of MeSO-differentiated HL-60 cells with 1 μM MK-886 prior to calcium ionophore challenge abolished leukotriene synthesis (Fig. 6C). This inhibition of leukotriene synthesis in MeSO-differentiated HL-60 cells was concentration-dependent with an IC50 of approximately 100 nM (Fig. 7).

DISCUSSION

In the present investigation we have shown that only cells known to express 5-lipoxygenase and to produce leukotrienes contain significant quantities of FLAP. Leukocytes have been shown to be the richest source of FLAP, which correlates well with their capacity to produce LTB4 (4). While the B cell line OKT3 did not express FLAP, normal human B-cells may contain a small amount of this protein (Fig. 1). Little leukotriene production has been found in B-cells (4). In the present experiments the elutriated B-cells may contain up to 2% monocyte contamination and the latter cells may be the source of the FLAP measured.

Differentiation of HL-60 cells after exposure to MeSO provides a useful model system for studying the regulation of leukotriene synthesis. Previous studies had shown induction of 5-lipoxygenase at both the mRNA and protein level in MeSO-differentiated HL-60 cells (20, 34). The results of the present study show concurrent induction of both FLAP and 5-lipoxygenase during MeSO-induced HL-60 differentiation that correlates well with their increased leukotriene synthetic capacity. Leukotriene synthesis in this system is inhibited by MK-886 in a concentration-dependent manner, with an IC50 similar to that observed for MK-886 inhibition of leukotriene synthesis in human leukocytes (11) and in FLAP/5-lipoxygenase transfected osteosarcoma cells (16). In human leukocytes MK-886 has been shown to inhibit and reverse 5-lipoxygenase translocation. In MeSO-differentiated HL-60 cells approximately 50% of the cytosolic 5-lipoxygenase has been previously shown to translocate to a membrane fraction after calcium ionophore challenge (34). This 5-lipoxygenase translocation is inhibited 70-90% by preincubation of the cells with 1 μM MK-886 (34). How MK-886 binding to FLAP prevents 5-lipoxygenase membrane association and activation is presently unclear. The cellular products of 5-lipoxygenase activation are predominately leukotrienes derived from LTA4 rather than 5-HETE and 5-HPETE, which are the major products observed for the isolated enzyme. Therefore, it appears that the activation of 5-lipoxygenase in response to a cellular stimulation permits the concerted oxygenation and dehydration reactions, which are known to be intrinsic to the enzyme, leading to the formation of LTA4. Such a process may be facilitated by direct or indirect membrane association of 5-lipoxygenase with FLAP.

The concurrent induction of FLAP and 5-lipoxygenase mRNAs in HL-60 cells suggests that their genes may respond to similar transcriptional regulation. In this regard it is somewhat surprising that the putative promoter region of the human 5-lipoxygenase gene (35) is similar to that of housekeeping genes that are constitutively expressed with little regulation (36). In another model myeloid system, namely transmutant-transformed macrophages, transcriptional control of the 5-lipoxygenase pathway has been shown to be a regulated and early event in macrophage differentiation (37). A comparison of the FLAP and 5-lipoxygenase promoter regions may yield interesting information on common transcriptional regulatory elements for expression of these proteins.

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