0026-895X/92/061014-06$03.00/0

MOLECULAR PHARMACOLOGY, 42:1014-1019

Cross-Species Comparison of 5-Lipoxygenase-Activating Protein

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Received June 30, 1992; Accepted September 3, 1992

SUMMARY

To identify regions of 5-lipoxygenase-activating protein (FLAP) important for the function of the protein and the binding of leukotriene biosynthesis inhibitors, we performed a cross-species analysis of FLAP. FLAP from all 10 mammalian species analyzed (human, monkey, horse, pig, cow, sheep, rabbit, dog, rat, and mouse) were immunologically cross-reactive and specifically bound leukotriene biosynthesis inhibitors with high affinity. Using the polymerase chain reaction, cDNA clones for FLAP from six species (monkey, horse, pig, sheep, rabbit, and mouse) were isolated and sequenced. The deduced amino acid sequences of FLAP show a high degree of identity to each other and to the published sequences for human and rat FLAP. Two regions of the protein are almost totally conserved among all of the species analyzed. This suggests that these regions have functional significance and may be involved in inhibitor binding.

LTs are potent mediators of a number of biological responses, including airway smooth muscle contraction and leukocyte aggregation (1). Increased levels of LTs in clinical samples implicate these compounds in a number of hypersensitivity and inflammatory diseases, including asthma and inflammatory bowel disease (reviewed in Refs. 2–4). Specific inhibitors of LT biosynthesis are consequently being developed for the treatment of these disorders.

The first two steps in the biosynthesis of LTs are catalyzed by the Ca^{2+}- and ATP-dependent enzyme 5-LO (5, 6), which also requires FLAP for cellular activity (7). The first step in the reaction involves the oxygenation of arachidonic acid to 5-HPETE, which is followed by the conversion of 5-HPETE to LTA_4. LTA_4 can subsequently be converted to LTB_4 by LTA_4 hydrolase or to LTC_4, LTD_4, and LTE_4 in a series of enzyme-catalyzed reactions (8).

The Ca^{2+}-dependent translocation of 5-LO from the cytosolic to a membrane fraction appears to be a critical step in the activation of the enzyme (9–11). Indole and quinoline classes of LT biosynthesis inhibitors and a series of structural hybrids of these compounds, termed quindoles, have been described that block this membrane association but have no significant inhibitory effect on 5-LO in cell-free assays. MK-886 (12, 13) and MK-0591 (14) are potent members of the indole and quindole classes of inhibitors, respectively. A photoaffinity ligand and affinity columns based on MK-886 were used to identify FLAP as the cellular target of this class of inhibitors (15). These results suggest that FLAP may function by regulating the association of 5-LO with the membrane, from which it derives the arachidonate used for LT biosynthesis. Recently a radioligand binding assay has been used to demonstrate that a correlation exists between the ability of compounds to bind to FLAP and to inhibit LT synthesis (16).

cDNA clones for FLAP have been isolated from human dimethylsulfoxide-differentiated HL-60 and rat RBL-1 cell cDNA libraries (7). The deduced amino acid sequences of rat and human FLAP correspond to hydrophobic proteins of 161 residues (18 kDa), with three potential membrane-spanning domains. Site-directed mutagenesis studies have localized regions of the protein that can be deleted without affecting the binding of LT biosynthesis inhibitors (17).

Because LT biosynthesis inhibitors are currently being evaluated in models of inflammation in a number of species, a knowledge of the primary sequence of FLAP and the affinity of inhibitors for FLAP from these species may aid in the interpretation of results obtained in these models. In the present study we have isolated and sequenced cDNAs from six mammalian species and compared these sequences with those

ABBREVIATIONS: LT, leukotriene; 5-HPETE, (5*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LTA_4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid; LTC_4, (5*S*)-hydroxy-(6*R*)-*S*-glutathionyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; LTD_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinylglycyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; LTE_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; MK-886, 3-[1-(*p*-chlorophenyl)-5-isopropyl-3-*t*-butylthio-1*H*-indol-2-yl]-2,2-dimethylpropanoic acid (formerly designated L-663,536); MK-0591, 3-[1-(4-chlorobenzyl)-3-(*t*-butylthio)-5-(quinolin-2-yl-methoxy)-indol-2-yl]-2,2-dimethylpropanoic acid; L-691,678, 3-[(4-azidophenyl)sulfonyl]-2-[(4-iodophenyl)methyl]-α,α-dimethyl-5-(2-quinolinylmethoxy)-1*H*-indole-2-propanoic acid; L-691,831, (1-(4-iodobenzyl)-3-(3,3-dimethyl-1-oxo-1-butyl)-5-quinol-2-ylmethoxy)-indol-2-yl)-2,2-dimethyl propionic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; IC_{50}, concentration required for 50% inhibition.

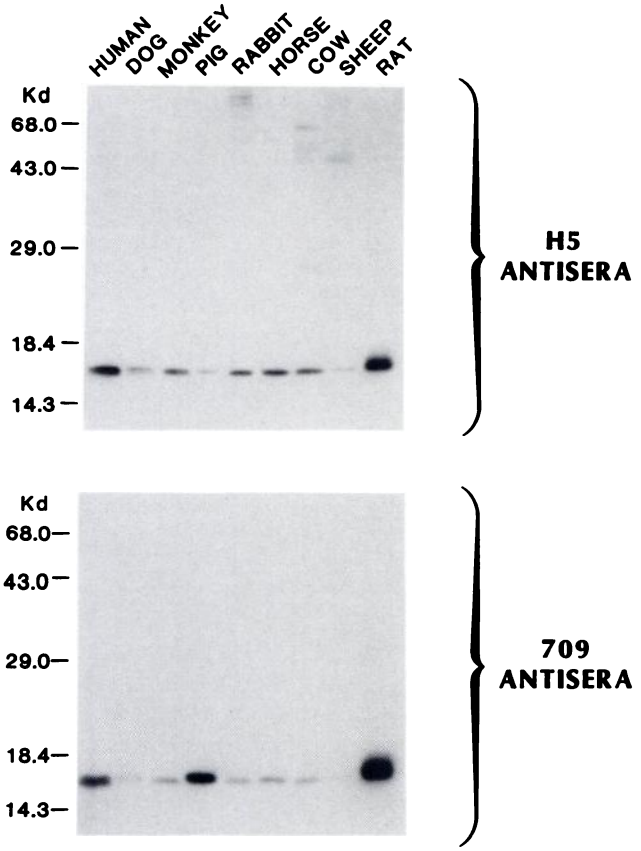


Fig. 1. Immunoblot analysis of FLAP. Membrane preparations of cells (40 μg) from the indicated species were analyzed by immunoblot as described in Experimental Procedures, using antiserum 709 or H5. The migration positions of molecular weight standards are indicated.

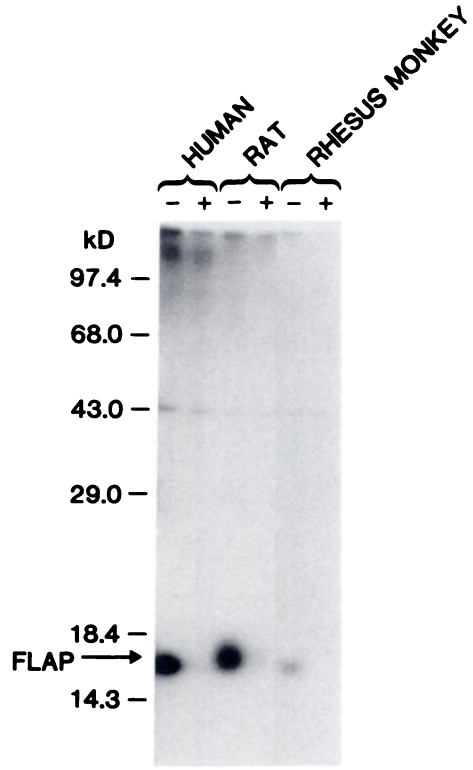


Fig. 2. Immunoprecipitation of photoaffinity-labeled FLAP. Photoaffinity labeling of membrane preparations (250 μg) from cells of the indicated species was performed as previously described (21), using ^{125}I-L-691,678. Labeling was carried out in the presence of dimethylsulfoxide vehicle (–) or 1 μM MK-886 (+). Immunoprecipitation was performed using antiserum H5. Immunoprecipitated proteins were analyzed by SDS-PAGE followed by autoradiography, as described in Experimental Procedures. The migration positions of molecular weight standards are indicated.

of human and rat FLAP. This analysis demonstrates that FLAP is highly conserved between mammalian species. Analysis of leukocyte membrane preparations by photoaffinity labeling and in a radioligand binding assay demonstrates that FLAP from each of these species specifically binds LT biosynthesis inhibitors with high affinity. The cross-species analysis of FLAP amino acid sequences complements site-directed mutagenesis studies to indicate amino acids that may play a critical role in inhibitor binding. Furthermore, the localization of regions of FLAP that are evolutionarily conserved suggests regions of the protein that are likely to have functional importance in the activation of 5-LO.

Experimental Procedures

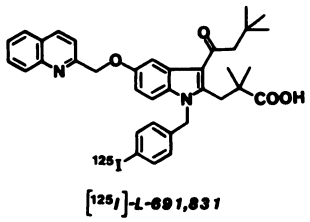
Cell preparation and subcellular fractionation. Blood samples (50–100 ml) from human, rhesus monkey, dog, horse, cow, pig, and rabbit were collected into heparinized tubes on ice. Leukocytes were isolated from these samples by dextran sedimentation, and contaminating erythrocytes were lysed as previously described (18). Because this procedure is not suitable for isolating sheep leukocytes, heparinized sheep blood (50 ml) was layered onto 37.5 ml of Ficoll-Paque (Pharmacia, Baie D'Urfe, Quebec, Canada) and then centrifuged at 450 × g for 30 min at 18°. The buffy coat obtained from this procedure was resuspended in 20 ml of Dulbecco's phosphate-buffered saline (GIBCO, Mississauga, Ontario, Canada) and centrifuged at 250 × g for 10 min. Contaminating erythrocytes were then lysed and leukocytes were frozen as described above. The P388D$_1$ mouse macrophage cell line was obtained from the American Type Culture Collection (Rockville, MD) (catalogue number TIB 63) and cultured in the recommended medium. Cells were washed twice and scraped into ice-cold phosphate-buffered saline. Cells were then harvested by centrifugation at 500 × g for 3 min at 4° and were stored at −80°.

For analysis in the FLAP binding assay, cell pellets were resuspended 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 disrupted by sonication (3 × 15 sec) using a Kontes microultrasonic cell disrupter (Kontes, Vineland, NJ). The cell homogenate was centrifuged at 100,000 × g for 60 min, and the resulting membrane pellet was resuspended in homogenization buffer to a protein concentration of approximately 5 mg/ml.

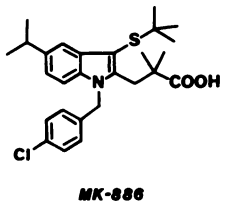
Immunoblot analysis. Membrane fractions (40 μg) were separated by 13.5% SDS-PAGE, according to the method of Laemmli (19), and were electrophoretically transferred to nitrocellulose for 16 hr at 100 mA using a Transblot apparatus (Bio-Rad, Burlington, Ontario, Canada) according to the manufacturers instructions. Immunoblot analysis was then performed as previously described (20), using a 1/200 dilution of rabbit polyclonal antipeptide antiserum and ^{125}I-Protein A (NEN-DuPont, Mississauga, Ontario, Canada) as the detection system. The antipeptide antisera used in these studies, designated 709 and H5, recognize amino acid residues 1–39 and 41–52 of FLAP, respectively (15, 21).

Photoaffinity labeling and immunoprecipitation. Membrane preparations were photoaffinity labeled with ^{125}I-L-691,678 and immunoprecipitated with antiserum H5 as described previously (21). Immunoprecipitated proteins were separated by 13.5% SDS-PAGE, and dried gels were exposed to X-ray film for 72 hr at −80°.

FLAP binding assay. Competitive filter binding assays were per-



[^{125}I]-L-691,831



MK-886

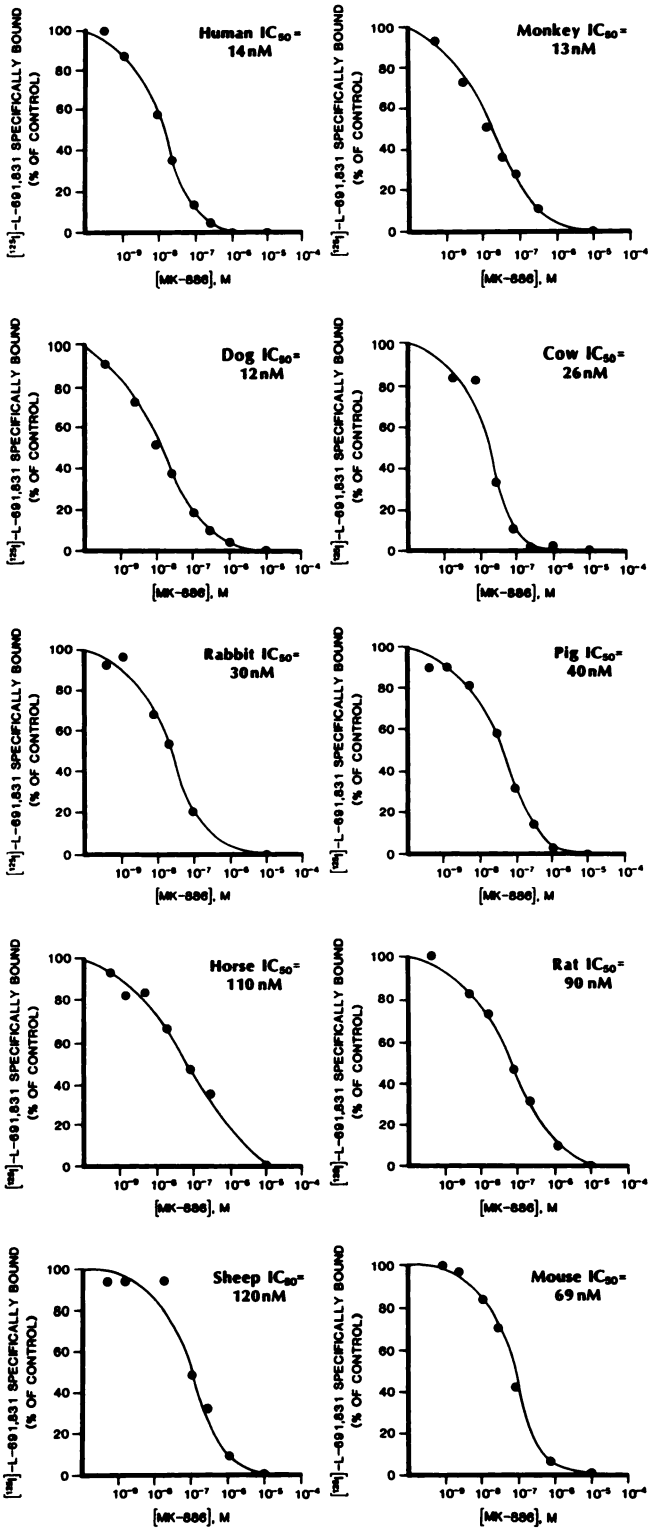


TABLE 1

Nucleotide sequence identity of FLAP from seven mammalian species, compared with human FLAP

GENBANK accession numbers are given for FLAP from the six species determined in this study.

	Nucleotide sequence homology to human	Accession number
	%	
Monkey	96.5	M96553
Horse	90.0	M96552
Rabbit	88.9	M96556
Pig	88.2	M96555
Sheep	87.1	M96557
Mouse	85.6	M96554
Rat	85.0	

formed in duplicate with the LT biosynthesis inhibitor ^{125}I-L-691,831, as described elsewhere (16), using approximately 15 μg of protein/assay and MK-886 as the competing ligand. IC_{50} values were computed using EBDA software; IC_{50} is defined as the concentration at which there is 50% inhibition of specific ^{125}I-L-691,831 binding.

PCR. Oligonucleotide primers were synthesized using a model 380B DNA synthesizer (Applied Biosystems, Foster City, CA). Primers used in the amplification of FLAP from rhesus monkey, horse, pig, rabbit, and mouse were as follows: 5′-TCTCTGGGGAGCCTGANGCAANCATG-3′ [F5′(−23)] and 5′-AGGGNATGAGAAGNAGNGGGGAGAT-3′ [F3′(484)]. Primers used in the amplification of FLAP from sheep were as follows: 5′-GCAANCATGGATCAAGANNCTGT-3′ [F5′(−6)] and 5′-AGGGNATGAGAAGNAGNGGGGAGAT-3′ [F3′(484)].

FLAP cDNAs for rhesus monkey, horse, and pig were amplified from 50–100 ng of poly(A)$^+$ RNA isolated from leukocytes using the Quick-prep mRNA purification kit (Pharmacia, Baie D'Urfe, Quebec, Canada). Mouse FLAP cDNA was amplified from total RNA (1 μg) prepared from P388D_1 cells. Sheep and rabbit FLAP cDNAs were amplified from approximately 1–9 × 10^6 phage from lung λgt10 cDNA libraries (Clontech, Palo Alto, CA).

For PCR amplification of FLAP from RNA samples, random hexamers were used as primers for the reverse transcriptase reaction (GeneAmp RNA PCR kit; Perkin Elmer, Rexdale, Ontario, Canada). The PCR cycling protocol was 1 min at 95° and 1 min at 55° for 35 cycles. For PCR amplification of FLAP from the cDNA libraries, the cycling protocol was 30 sec at 95°, 30 sec at 47, 52, or 55°, and 30 sec at 72° for 35 cycles. For each species, two separate PCR reactions were performed.

DNA fragments amplified by PCR were separated by low melting point agarose gel electrophoresis, visualized by ethidium bromide staining, excised from the gel, and isolated by sequential phenol, phenol/chloroform, and chloroform extractions. The fragments were then ligated into the pKS vector (Stratagene, La Jolla, CA), which had been previously digested with *Eco*RV and treated with *Taq* DNA polymerase (Perkin Elmer) in the presence of dTTP (22).

DNA sequencing. Plasmid DNA was prepared using the alkaline lysis method (23), followed by chromatography on nucleic acid chromatography (NACs) columns (GIBCO). DNA was sequenced using the dideoxy chain termination method with T7 DNA polymerase (United States Biochemical Corp., Cleveland, OH). DNA sequences were determined for two clones from independent PCR amplifications on both

Fig. 3. Radioligand binding analysis of FLAP. Radioligand binding assays were performed on membrane preparations from cells of the indicated species, as described in Experimental Procedures. Assays were carried out using the quindole ^{125}I-L-691,831 in the presence of the indicated concentrations of MK-886. The mean amount, from duplicate assays, of ^{125}I-L-691,831 specifically bound at each concentration of MK-886 is indicated, relative to that in the absence of MK-886. The calculated IC_{50} values are shown for each species. The structures of ^{125}I-L-691,831 and MK-886 are shown.

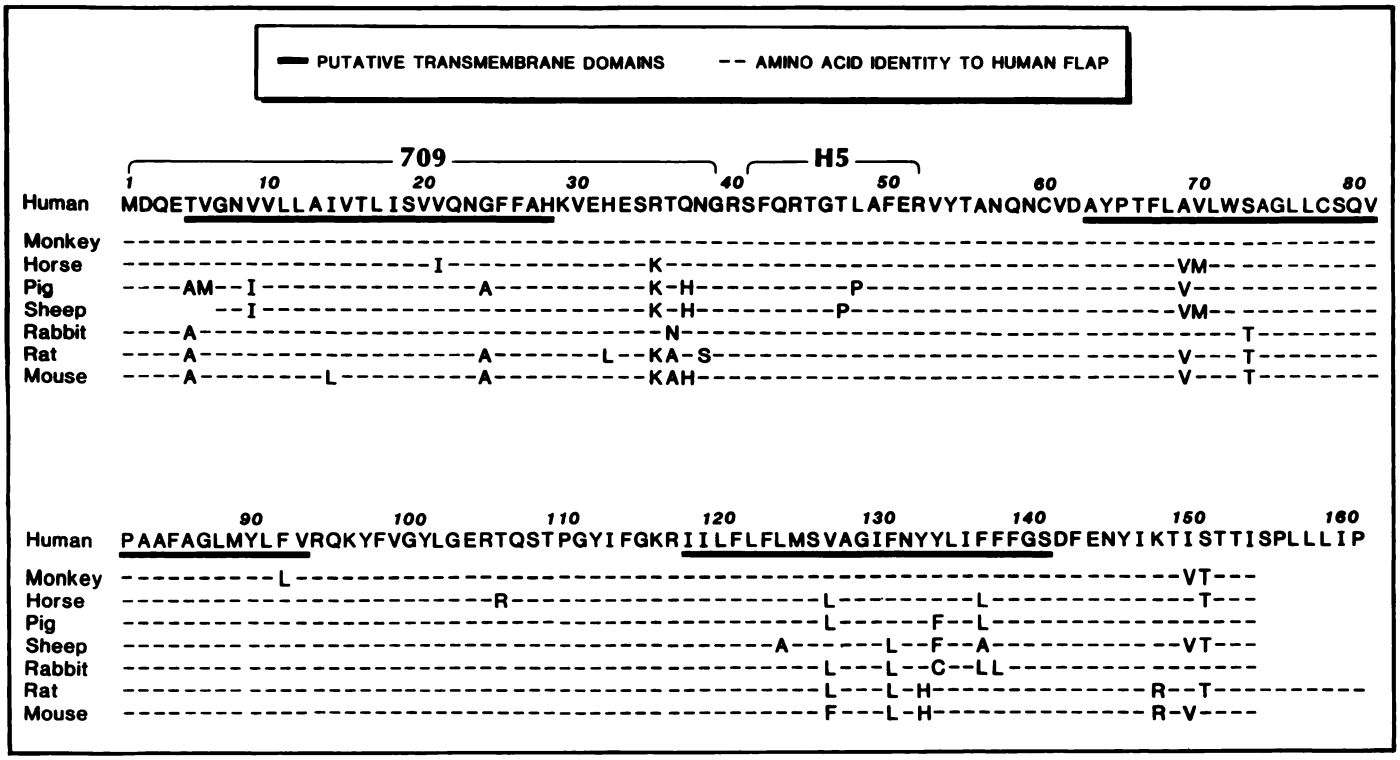


Fig. 4. Comparison of FLAP amino acid sequences. A comparison of the deduced protein sequences of FLAP from eight mammalian species is shown. Protein sequences are in standard single-letter amino acid code; *dashed lines*, identity with the human FLAP sequence. Regions of the FLAP amino acid sequence that are predicted to be membrane-spanning domains (15) and peptide sequences that are recognized by antisera 709 and H5 are indicated. Spaces in the sequences in the amino and carboxyl termini correspond to regions where primers used for PCR analysis annealed and, consequently, no sequence information is available. Human and rat sequences were from the literature (7).

strands using the vector-specific SK and KS sequencing primers (Stratagene). The sequences obtained were recorded, aligned, and analyzed using the University of Wisconsin Genetics Computer Group sequence analysis programs (24).

Results and Discussion

Immunological cross-reactivity of mammalian FLAP. FLAP has previously been detected immunologically in various rat, human, and mouse cells that synthesize LTs (20). As a first step in the cross-species analysis of FLAP, we compared FLAP expression in leukocytes from nine mammalian species by immunoblot. Two antisera, which recognize peptide sequences 1–39 and 41–52 of rat and human FLAP, specifically detected an 18-kDa protein corresponding to the migration position of FLAP in membrane preparations from human, dog, rhesus monkey, pig, rabbit, horse, cow, sheep, and rat leukocytes (Fig. 1), as well as from the mouse macrophage cell line P388D$_1$ (data not shown). Significant differences in the intensity of the signal were observed. For example, with pig FLAP the signal achieved with antiserum 709 was greater than that achieved with antiserum H5, whereas the opposite was the case with horse FLAP. These differences may be due to the amount of FLAP in the various preparations and/or the affinity of the antisera for the different FLAP molecules. The immunological detection of FLAP in these species suggested significant conservation of amino acid sequence in the regions recognized by the antipeptide antisera. The proline residues that occur in pig and sheep FLAP at positions 47 and 48, respectively (see below), may account for the lower signal observed with these species in the immunoblot using H5 antipeptide antiserum (Fig. 1), which recognizes amino acids 41–52 of FLAP.

Photoaffinity labeling and immunoprecipitation of FLAP. Immunoprecipitation studies have shown that human and rat FLAP specifically bind a radioactively labeled photoaffinity analogue of the indole series of LT biosynthesis inhibitors (15). Because antipeptide antisera detected FLAP from all species examined, we performed photoaffinity labeling and immunoprecipitation studies using the quindole ligand ^{125}I-L-691,678. After photoaffinity labeling of membrane preparations from human, rat, and rhesus monkey leukocytes, antiserum H5 immunoprecipitated a single radiolabeled 18-kDa polypeptide, corresponding to the molecular mass of FLAP (Fig. 2). Labeling of FLAP from each of these species was competed for by preincubation with MK-886. Similar results were obtained with membrane preparations from cells of the other species analyzed by immunoblot (data not shown). This demonstrates that FLAP from each of these species specifically binds LT biosynthesis inhibitors.

Binding of LT biosynthesis inhibitors to FLAP. To quantitate the affinity of FLAP from various species for LT biosynthesis inhibitors, membrane preparations were analyzed in a FLAP binding assay. This assay utilizes the radioiodinated quindole inhibitor ^{125}I-L-691,831 and has been used previously to assess the affinity of human FLAP for different classes of LT biosynthesis inhibitors (16). MK-886 has an IC_{50} value of approximately 20 nM for human FLAP in the FLAP binding assay (16).[1] Using the same membrane preparations analyzed

[1] S. Charleson, unpublished observations.

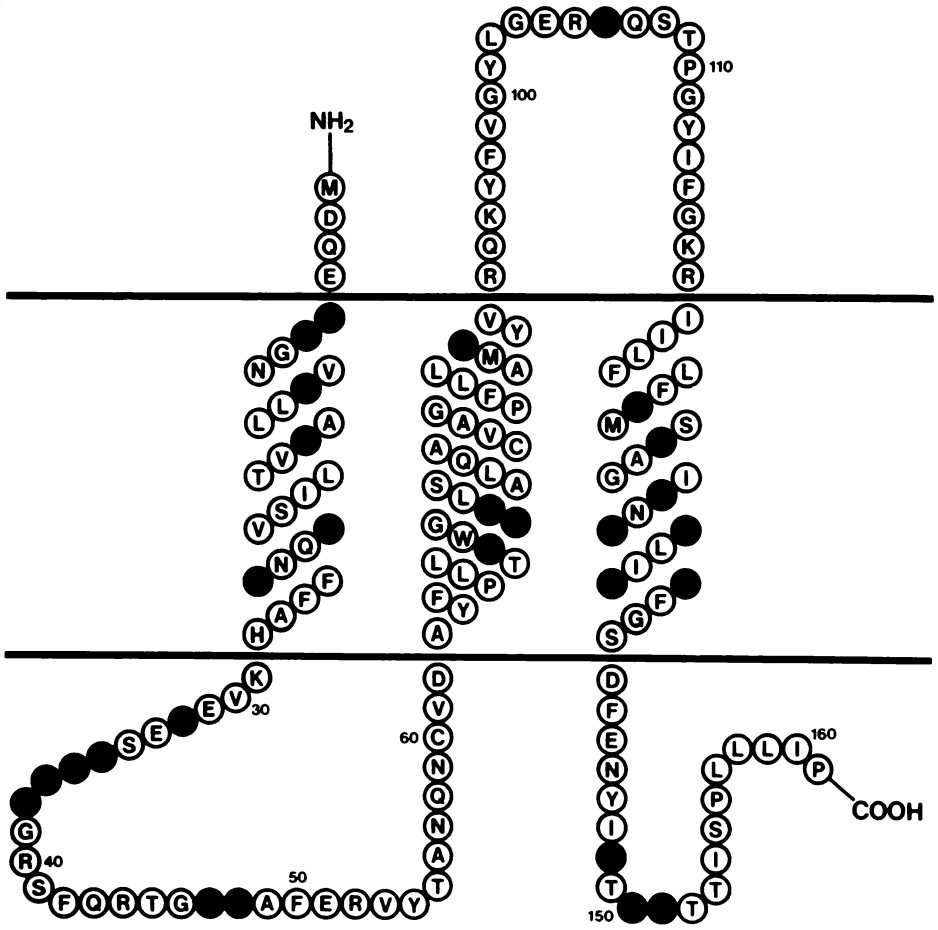


Fig. 5. Proposed membrane topology of FLAP. The proposed membrane topology of FLAP based on hydropathy analysis (7) is indicated; *horizontal lines*, membrane boundaries. The standard single-letter amino acid code is used. ●, Residues that differ from human FLAP in one or more of the seven species analyzed.

by immunoblot (Fig. 1), human FLAP had an IC_{50} value for MK-886 of 14 nM (Fig. 3). Under similar assay conditions, IC_{50} values for MK-886 with membrane preparations from other species (rhesus monkey, dog, cow, rabbit, pig, horse, sheep, rat, and mouse) ranged from 12 nM to 120 nM (Fig. 3). These results demonstrate that a high affinity inhibitor-binding domain is present in FLAP from each of these species. This is consistent with previous demonstrations that LT biosynthesis inhibitors that specifically bind to FLAP are effective *in vivo* in a variety of mammalian species (12, 14).

Cloning and sequencing of FLAP cDNAs. To determine the extent of the similarity between FLAP from various species at the amino acid level, we cloned by PCR and sequenced FLAP cDNAs from rhesus monkey, horse, pig, sheep, rabbit, and mouse. Sequences of FLAP PCR primers were selected on the basis of nucleotide sequence similarity proximal to the initiation and termination codons of the cDNAs for rat and human FLAP (7). The primer pair F5′(−23)/F3′(484) amplified the expected 507-base pair DNA fragment corresponding to FLAP from all species examined except sheep. Sheep FLAP could not be amplified using the aforementioned primer pair but was amplified with the F5′(−6)/F3′(484) primer pair, resulting in the expected 490-base pair DNA fragment. Primers F5′(−6) and F3′(484) overlap codons corresponding to the six amino-terminal and seven carboxyl-terminal amino acids, respectively. The identity of the amplified DNA as FLAP was confirmed by Southern blot analysis using human FLAP cDNA as a hybridization probe (data not shown). FLAP cDNAs sequenced from rhesus monkey, horse, pig, sheep, rabbit, and mouse were shown to exceed 84% identity to the human sequence (Table 1).

Comparison of deduced amino acid sequences of FLAP. The alignment of the amino acid sequences deduced from the FLAP cDNA sequences from eight species is shown in Fig. 4. Comparison of these sequences shows 82% identical residues among all eight species, with all differences being restricted to only 28 positions. According to the criteria of Dayhoff *et al.* (25), conservative substitutions occur in 13 of the 28 positions. For example, the arginine at residue 35 in human FLAP is a lysine in five of the seven other species analyzed, and the serine at position 151 in human FLAP is a threonine in four of the seven species. The percentage of amino acid sequence identity to human FLAP was as follows: monkey, 98%; horse, 95%; rabbit, 95%; pig, 93%; mouse, 92%; rat, 92%; and sheep, 92%. All the sequence differences are amino acid substitutions; there are no amino acid insertions or deletions.

The proposed membrane topology of FLAP based on hydropathy analysis consists of three transmembrane domains separated by two hydrophilic loops, with the amino and carboxyl termini on opposite sides of the membrane (15) (Fig. 5). The membrane orientation of FLAP and the cellular membrane to which the protein is localized are not yet known. Sequence comparison reveals that within two regions of the protein, a 30-amino acid stretch (residues 39–68) and a 50-amino acid stretch (residues 74–123), only four amino acid substitutions

occur among all eight species. These two highly conserved sequences are located within the putative extramembrane loops and the second transmembrane domain. This suggests that these regions of the protein play critical roles in the function of FLAP. Furthermore, the finding that FLAP from each of these species specifically binds LT biosynthesis inhibitors suggests that amino acids within these regions are also involved in inhibitor binding.

The amino acid sequence at the amino-terminal half of the loop between the first and second transmembrane domains (residues 32–38) was not conserved between the species analyzed (Fig. 4). This suggests that these amino acids do not play a critical role in the function of the protein. Site-directed mutagenesis has shown that deletions in this region of the protein do not significantly affect binding of LT biosynthesis inhibitors to FLAP (17). In contrast, deletion of amino acids in the carboxyl-terminal half of this loop (residues 52–62), which we have shown to be conserved among all species analyzed, results in a loss of inhibitor binding (17). The cross-species sequence analysis presented in this study indicates residues for future mutagenesis studies to determine which amino acids are responsible for the subtle differences in the affinity of FLAP for inhibitors that we observed (Fig. 3). For example, horse and sheep FLAP have IC_{50} values for MK-886 greater than 100 nM and are the only species to have methionine residues at position 70. It will be interesting to determine whether mutations at this residue alter the IC_{50} for MK-886 exhibited by human FLAP.

These studies demonstrate that FLAP from different mammalian species have highly conserved regions at the amino acid level and have similar affinities for LT biosynthesis inhibitors. The roles that amino acid residues in these regions play in the activation of 5-LO and the binding of LT biosynthesis inhibitors are currently under investigation.

Acknowledgments

The authors would like to thank M. Cox, R. Schaum, and I. Foltz for technical assistance, W. Grzywacz for providing blood samples, B. Kennedy for providing RNA from the P388D_1 cell line, and P. Tagari and W. M. Abraham for the procedure to isolate sheep leukocytes.

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