0026-895X/92/010094-09$03.00/0

MOLECULAR PHARMACOLOGY, 42:94-102



# Identification of Amino Acid Residues of 5-Lipoxygenase-Activating Protein Essential for the Binding of Leukotriene Biosynthesis Inhibitors

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Received March 13, 1992; Accepted April 23, 1992

## SUMMARY

5-Lipoxygenase-activating protein (FLAP) is specifically labeled by [$^{125}$I]L-669,083 and [$^{125}$I]L-691,678, photoaffinity analogues of two classes of potent leukotriene biosynthesis inhibitors. Because human FLAP contains only a single tryptophan residue at position 72 and two internal methionine residues at positions 89 and 125, we have used reagents that specifically cleave at these residues, in conjunction with antipeptide antisera, to localize the site of attachment of the photoaffinity ligands. Immunoprecipitation of specifically labeled peptide fragments after digestion of photoaffinity-labeled FLAP by iodosobenzoic acid at $^{72}$Trp demonstrates that the inhibitors bind to FLAP amino-terminal to this residue. This finding is consistent with similar immunoprecipitation studies after digestion at methionine residues using cyanogen bromide. These findings localize the site of attachment of the inhibitors to a region of FLAP that includes the hydrophilic loop between the proposed first and second transmembrane regions. Based on these findings, site-directed mutagenesis of human FLAP was performed to define key amino acids involved in inhibitor binding. Using a radioligand binding assay, analysis of mutants of human FLAP expressed in COS-7 cells demonstrates that a number of residues in the amino-terminal half of the first hydrophilic loop of the protein can be deleted without significantly affecting inhibitor binding. In contrast, no inhibitor binding was detectable with mutants in which amino acid residues in the carboxyl-terminal half of this loop were deleted. Furthermore, a point mutation of $^{62}$Asp to asparagine results in a mutant with dramatically reduced affinity for inhibitors. This loss of affinity was not displayed by a mutant in which $^{62}$Asp was mutated to a glutamate residue, suggesting that a negative charge associated with residue 62 may be critical for inhibitor binding. The roles that amino acid residues in the carboxyl-terminal half of the first hydrophilic loop of FLAP may play in the binding of leukotriene biosynthesis inhibitors are currently under investigation.

The initial step in the biosynthesis of leukotrienes involves the calcium-dependent translocation of the enzyme 5-LO from the cytosolic to a membrane fraction (1–3). After membrane association, 5-LO catalyzes a two-step reaction that involves oxygenation of arachidonate to 5-HPETE, followed by the conversion of 5-HPETE to $LTA_4$ (4, 5). $LTA_4$ can subsequently be converted to $LTB_4$ by the enzyme $LTA_4$ hydrolase or to the peptidoleukotrienes $LTC_4$, $LTD_4$, and $LTE_4$ in a series of enzyme-catalyzed reactions (6).

The potent and diverse biological effects of leukotrienes, coupled with the measurement of increased levels of leukotrienes in various disease states, implicates these compounds in a number of inflammatory and hypersensitivity disorders, including asthma, inflammatory bowel disease, and endotoxin shock (7–11). Specific inhibitors of leukotriene biosynthesis may, therefore, provide a novel therapy for these disorders (12–16). Three classes of potent inhibitors of cellular leukotriene biosynthesis, based on indole, quinoline, and quinoline-indole hybrid structures (termed quindoles), have recently been shown to inhibit specifically the membrane association of 5-LO (17–19). Radioiodinated photoaffinity analogues of the indole and quindole series of inhibitors (18–21) and a radioligand binding

**ABBREVIATIONS:** 5-LO, 5-lipoxygenase; 5-HPETE, (5*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; $LTA_4$, 5,6-oxido-7,9,11,14-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; FLAP, 5-lipoxygenase-activating protein; hPMN, human polymorphonuclear leukocytes; MK-886, 3-[1-(*p*-chlorophenyl)-5-isopropyl-3-*tert*-butylthio-1*H*-indol-2-yl]-2,2-dimethylpropanoic acid (formerly designated L-663,536); L-689,037, (1-(4-chlorobenzyl)-3-(3,3-dimethyl-1-oxo-1-butyl)-5-quinol-2-ylmethoxy)-indol-2-yl)-2,2-dimethyl propionic acid; L-669,083, 3-(1-(4-hydroxy-3-iodobenzyl)-3-(4-azidophenylsulfonyl)-5-isopropylindol-2-yl)-2,2-dimethylpropanoic acid; L-691,678, 3-[(4-azidophenyl)sulfonyl]-2-[(4-iodophenyl)methyl]-$\alpha$,$\alpha$-dimethyl-5-(2-quinolinylmethoxy)-1*H*-indole-2-propanoic acid; L-674,573, 2-((4-(1-thioacetic acid-4-phenylbutyl)phenoxy)methyl)quinoline; REV-5901, 3-(1-hydroxypentyl)-1-(quinoline-2-ylmethoxy)benzene; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IBA, iodosobenzoic acid; hFLAP, human 5-lipoxygenase-activating protein.

assay utilizing the quindole [$^{125}$I]L-691,831 (22) have been used to show that members of these three series of inhibitors specifically interact with FLAP, an 18-kDa integral membrane protein that has recently been isolated from rat and human neutrophils (20). Furthermore, a correlation exists between the ability of compounds in these series to bind to FLAP and to inhibit leukotriene synthesis in hPMN (18, 21, 22). These findings are consistent with a model for leukotriene biosynthesis in which 5-LO specifically interacts at the membrane with FLAP or a membrane component whose activity is regulated by FLAP.

Transfection experiments in osteosarcoma cells, using full length cDNA molecules for FLAP and 5-LO, have demonstrated that expression of both of these proteins is essential for cellular leukotriene synthesis (23). However, the mechanism by which FLAP activates 5-LO remains unclear. Whereas studies utilizing inhibitors of FLAP suggest that FLAP itself has no phospholipase activity,[1] it is possible that FLAP could stimulate a phospholipase involved in liberating arachidonate for leukotriene synthesis. Alternatively, FLAP may interact directly with 5-LO at the membrane, to optimize the efficient utilization of arachidonate, present in phospholipid stores, as a substrate for leukotriene synthesis. If FLAP does interact directly with 5-LO, leukotriene biosynthesis inhibitors may compete with 5-LO for a common binding site on the protein.

In the present studies, we have utilized two approaches to localize amino acid residues of FLAP involved in the binding of leukotriene biosynthesis inhibitors. Initially, reagents that specifically cleave polypeptide chains at methionine and tryptophan residues were used, in conjunction with polyclonal antisera that recognize defined and nonoverlapping peptide sequences of FLAP, to localize the region of FLAP to which photoaffinity analogues of leukotriene biosynthesis inhibitors bind. Results from these studies are consistent with the ligands binding to FLAP amino-terminal to $^{72}$Trp. This finding provided a focus for site-directed mutagenesis studies, which defined specific amino acids in the first hydrophilic loop of FLAP that are essential for inhibitor binding. These results provide a basis for future studies to define the roles that specific amino acid residues of FLAP play in binding leukotriene biosynthesis inhibitors and whether such an inhibitor binding site also represents a site of interaction between FLAP and 5-LO.

## Materials and Methods

**Human leukocyte preparation and subcellular fractionation.** hPMN were prepared from buffy coat concentrates by dextran sedimentation and hypotonic lysis of contaminating red blood cells, as previously described (24). The 0–30% ammonium sulfate fraction from the 10,000 × *g* supernatant of leukocyte homogenates was prepared according to previously reported procedures (24) and stored at −70° before use. This fraction has been shown to include membranes containing FLAP (20).

**Photoaffinity labeling.** Photoaffinity labeling was performed essentially as previously described (25). Protein fractions were incubated in the presence of 1 μM MK-886 or dimethylsulfoxide vehicle for 2 min at 37°, in 200 μl of 50 mM Tris·HCl, pH 7.4, 25 mM EDTA, 20% glycerol (buffer A). Samples were then incubated for an additional 2 min at 37° with either [$^{125}$I]L-669,083 ($2 \times 10^6$ cpm; 500–1000 Ci/mmol) or [$^{125}$I]L-691,678 ($1 \times 10^6$ cpm; 500–1000 Ci/mmol), followed by exposure to UV light (Hanovia 450 W lamp suspended 15 cm above samples) for 3 min at room temperature.

**Immunoprecipitation.** Protein samples (in 500 μl of buffer A) were mixed with 150 μl of 1% SDS, 100 mM Tris·HCl, pH 8.0, and incubated for 15 min at room temperature, with shaking. Samples were then heated at 95° for 5 min, followed by the addition of 0.65 ml of 2% Triton X-100 (v/v), 600 mM NaCl, 20 mM Tris·HCl, pH 7.2 (2× IP buffer), and incubation at 4° for 30 min, with shaking. After centrifugation at 15,000 × *g* for 15 min at 4° to remove nonspecific precipitates, 50 μl of antiserum and 4 mg of Protein A-Sepharose (Pharmacia-LKB, Baie D'Urfe, Quebec, Canada) were added to the samples, and the resulting mixture was incubated at 4° for 60 min, with shaking. The resulting immunoprecipitate was pelleted by centrifugation at 15,000 × *g* for 15 sec and washed twice with IP buffer and twice with 10 mM Tris·HCl, pH 7.0. Proteins were separated from the Protein A-Sepharose complex by heating at 95°, in SDS-PAGE sample buffer (25), for 5 min and were separated on polyacrylamide gels in the presence of SDS, according to the method of Laemmli (26). Gels were dried and exposed to Kodak XAR-2 film at −70° for 24–72 hr, and the amount of radioactivity associated with polypeptides was determined by densitometry, using a model 300A scanning laser densitometer (Molecular Dynamics, Sunnyvale, CA). Polyclonal antipeptide antisera used in these studies have been previously described (18, 20, 21, 25). Antisera H5 and H9 recognize amino acid residues 41–52 and 101–118 of FLAP, respectively (Fig. 1). Antiserum 709 (Fig. 1) was raised to a fusion protein of the amino terminus of the *Escherichia coli* CheY protein with the first 39 amino acid residues of FLAP (20).

**Treatment with IBA or cyanogen bromide.** Membrane preparations from human leukocytes (1 mg of protein in buffer A) were labeled with [$^{125}$I]L-669,083 or [$^{125}$I]L-691,678 as described above. Samples were then treated with 4 mg of IBA (Pierce, Rockford, IL) or 15 mg of CNBr (Pierce), in the presence of 80% acetic acid, for 24 hr at room temperature, in a final volume of 1 ml. For incubations with IBA, 4 M guanidine-HCl was also added to the reaction mixture. After treatment with these reagents, samples were dialyzed at 4° against 2 liters of 50 mM Tris·HCl, pH 7.5, lyophilized, and resuspended in 500 μl of buffer A. Samples were then immunoprecipitated as described above and separated by SDS-PAGE. After electrophoresis, gels were fixed, dried, and exposed to Kodak XAR-2 film at −70° for 3–7 days.

**Mutagenesis and expression vectors.** All general recombinant DNA procedures were performed as previously described (27). The nucleotide sequences of the cDNA molecules for rat FLAP and hFLAP have previously been published (23, 28). The hFLAP cDNA, obtained from R. Dixon, was subcloned into the multiple cloning site of Bluescript (Stratagene, La Jolla, CA), to yield the plasmid pBShFLAP. Single-stranded DNA was prepared from pBShFLAP using the Amersham oligonucleotide-directed mutagenesis kit (Amersham, Arlington Heights, IL), according to the manufacturers instructions. This kit was also used to introduce mutations into the hFLAP cDNA. Deletion mutants were made using oligonucleotides 30 nucleotides in length, containing sequences evenly flanking the nucleotides to be deleted. These mutants are designated hFLAP(del *A*-*B*), where *A* and *B* are the first and last amino acid of FLAP included in the deleted region. Single amino acid changes were introduced using appropriate oligonucleotides 23 nucleotides in length, with one or two nucleotide mismatches. These mutants are designated hFLAP($^x$*A*-*B*), where *x* represents the position of the amino acid residue in the sequence of FLAP, *A* represents the amino acid residue in hFLAP, and *B* represents the residue to which the amino acid was changed. Oligonucleotides were synthesized using the 380B DNA synthesizer (Applied Biosystems, Mississauga, Ontario, Canada). Introduction of the desired mutation was confirmed by dideoxy sequencing, using a T7 sequencing kit (Pharmacia, Baie D'Urfe, Quebec, Canada). hFLAP and FLAP mutants were excised from pBShFLAP and subcloned into the multiple cloning site of pEUK-C1 (Clontech, Palo Alto, CA), to yield the expression vector pEUKhFLAP. This expression vector contains an SV40 late promoter suitable for transient, high level, expression in COS cells.

[1] D. K. Miller, E. Ham, and D. Soderman, unpublished observations.

**Mammalian cell expression.** COS-7 cells were obtained from the American Type Culture Collection (Rockville, MD) and grown in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) containing glucose (4.5 g/liter), sodium pyruvate (110 mg/liter), L-glutamine (0.584 g/liter), penicillin (5000 units/ml), and streptomycin (5 g/liter) and supplemented with 10% fetal bovine serum (Sigma). Cells were maintained at 37° in an atmosphere of 5% $CO_2$/95% air. Twenty four hours before transfection, cells were trypsinized and approximately $10^7$ cells were plated into 500-$cm^2$ tissue culture plates (Nunc, Kamstrup, Denmark). Four hours before transfection, medium was changed. DNA (200 μg) was transfected into the monolayer cells using a Transfinity calcium phosphate transfection kit (BRL, Burlington, Ontario, Canada), according to the manufacturer's instructions. Twenty four hours after transfection, medium was changed, and the cells were incubated for an additional 72 hr.

**Membrane preparation from COS-7 cells.** After the transfection procedure described above, medium was aspirated from COS-7 cells, which were then washed with 2 × 10 ml of ice-cold phosphate-buffered saline. Cells were then scraped into 10 ml of ice-cold phosphate-buffered saline and centrifuged at 500 × *g* for 5 min. The resulting cell pellet was resuspended in 15 mM Tris·HCl, pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and cells were lysed by freeze-thawing twice in a dry ice/ethanol bath and sonication (3 × 15-sec bursts), using a 4710 series ultrasonic homogenizer (Cole-Parmer, Chicago, IL), set at 75% duty cycle, power level 3. Sonicated samples were centrifuged at 10,000 × *g* for 10 min, and the 10,000 × *g* supernatant was further centrifuged at 100,000 × *g* for 60 min. The 100,000 × *g* membrane pellets were resuspended by sonication for 2 × 5 sec in 50 mM Tris·HCl, 25 mM EDTA, 20% glycerol, pH 7.4, using a Microsonic cell disrupter (Kontes, Vineland, NJ). The 100,000 × *g* membrane preparation from human leukocytes was prepared as previously described (22). Protein samples were stored at a concentration of approximately 10 mg/ml, at −70°.

**Protein immunoblotting.** Protein samples (100 μg) were separated on 13.5% polyacrylamide gels in the presence of SDS, with a 3% stacking gel, according to the method of Laemmli (26). Samples were then transferred to nitrocellulose overnight at 100 mA, using a Trans-blot apparatus (Bio-Rad, Mississauga, Ontario, Canada), according to the manufacturer's instructions. Immunoblot analysis was performed as previously described (25), using a 1/250 dilution of antiserum and $^{125}$I-Protein A (NEN-DuPont, Mississauga, Ontario, Canada) as the detection system.

**Radioligand binding assays.** Filter binding assays were performed with the quindole leukotriene biosynthesis inhibitor [$^{125}$I]L-691,831, essentially as described previously (22). Reactions, performed in duplicate, were initiated by the addition of 100,000 × *g* membrane preparations from COS-7 cells (10–80 μg of protein) or human leukocytes (5–20 μg of protein), and the assay mixtures were incubated at room temperature for 20 min. Incubations were terminated by the addition of 4 ml of ice-cold 0.1 M Tris·HCl, pH 7.5, 0.05% Tween 20 (TT buffer), and immediately filtered through Whatman GF-B filters that had been presoaked in TT buffer. After rinsing of incubation tubes with two 4-ml aliquots of ice-cold TT buffer, filters were transferred to polystyrene tubes, and bound radioactivity was measured in an LKB 1272 Clinigamma Quatro counter (Pharmacia-LKB, Baie D'Urfe, Quebec, Canada). Competitive radioligand binding assays were performed by incubation of membranes with [$^{125}$I]L-691,831 in the presence of various concentrations of nonradioactive competing ligands, followed by the filter binding assay as described above. Specific binding was defined as the difference between the binding of [$^{125}$I]L-691,831 in the absence and presence of $10^{-5}$ M L-689,037, a quindole compound with an $IC_{50}$ of 2 nM for the inhibition of leukotriene biosynthesis in hPMN (21). Linearity of specific binding of [$^{125}$I]L-691,831 with protein levels used in this study was verified. Binding parameters were determined by computer fit, using the LIGAND program of Munson and Rodbard (29).

**Protein assay.** Protein concentrations were determined in microtiter plates, using Coomassie protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's instructions, with bovine serum albumin as a standard.

## Results

Radiolabeled photoaffinity analogues have been extensively used to to localize amino acid residues involved in the binding of ligands to membrane proteins (30–32). We have utilized analogues of two series of leukotriene biosynthesis inhibitors, in conjunction with FLAP antisera, to localize the binding site on human FLAP for these inhibitors. Analysis of the deduced amino acid sequence of FLAP suggests that the protein contains three transmembrane regions separated by two short hydrophilic loops, with the amino and carboxyl termini on opposite sides of the membrane (23). A model for this transmembrane topology and the location of the amino acid sequences recognized by the antipeptide antisera 709, H5, and H9 are shown in Fig. 1. These antisera have previously been shown to detect FLAP from human leukocytes, by immunoblot analysis (20, 21). The indole [$^{125}$I]L-669,083 and the quindole [$^{125}$I]L-691,678 photoaffinity ligands label a number of proteins in these membrane preparations (20, 21). However, only the labeling of FLAP is specifically inhibited by potent members of the indole, quinoline, and quindole series of inhibitors (18, 20, 21). Photoaffinity labeling of human leukocyte membrane preparations, followed by immunoprecipitation with antiserum 709, H5, or H9, resulted in the detection of a single radiolabeled species of approximately 18 kDa, corresponding to the migration position

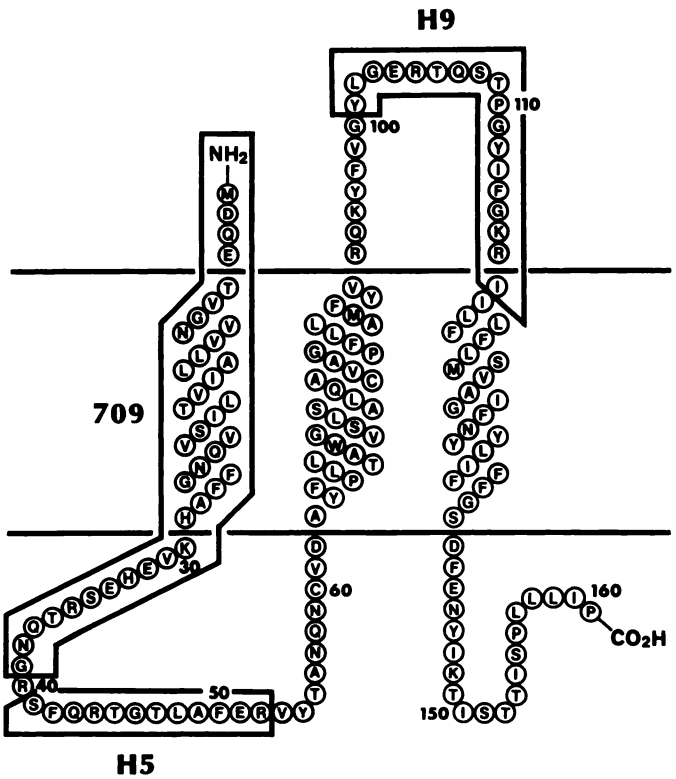


**Fig. 1.** Amino acid sequence and proposed transmembrane topology of FLAP. The deduced amino acid sequence of human FLAP and the membrane boundaries, based on hydropathy plot analysis, have been reported previously (23, 29). *Horizontal lines*, proposed limits of the lipid bilayer. The standard single-letter amino acid code is used. Antisera 709, H5, and H9 recognize amino acid residues 1–39, 41–52, and 101–118 (*boxes*), respectively.

of FLAP (Fig. 2). Preincubation with 1 μM MK-886 before photoaffinity labeling inhibited the labeling associated with this species, confirming its identity as FLAP. Immunoprecipitation results similar to those shown with [$^{125}$I]L-669,083 in Fig. 2 have been reported with [$^{125}$I]L-691,678 (21).

CNBr is well characterized for its ability to cleave polypeptide chains at methionine residues (33). The presence of only two internal methionine residues in human FLAP, at positions 89 and 125 (Fig. 1), therefore, makes CNBr a potentially useful reagent for analysis of functional domains of FLAP. The standard reaction conditions for CNBr digestion require the presence of strong acids such as HCl. Due to the lability of the radioactive photoaffinity ligands used in these studies under these conditions (data not shown), acetic acid was used to maintain a low pH in the reaction mixture. After photoaffinity labeling of FLAP with [$^{125}$I]L-669,083 and digestion with CNBr, samples were immunoprecipitated with antiserum 709, H5, or H9 (Fig. 3). All three of these antisera immunoprecipitated a photoaffinity-labeled species of approximately 18 kDa, corresponding to FLAP, which was not cleaved by CNBr under the reaction conditions used. In addition, 709 and H5, but not H9, immunoprecipitated a photoaffinity-labeled species that migrated with a molecular mass corresponding to approximately 7 kDa. Immunoprecipitation results similar to those obtained with [$^{125}$I]L-669,083 were obtained after digestion by CNBr of FLAP labeled with [$^{125}$I]L-691,678 (data not shown). Radioactivity

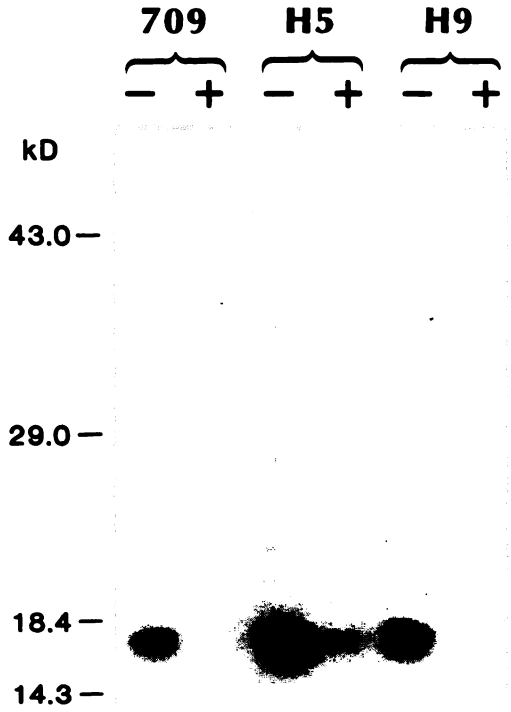


**Fig. 2.** Immunoprecipitation of photoaffinity-labeled FLAP. Human leukocyte membrane proteins were photoaffinity labeled and immunoprecipitated, as described in Materials and Methods. Samples were then separated by 13.5% SDS-PAGE, and dried gels were exposed to Kodak XAR-2 film at −70° for 2 days. Immunoprecipitation was performed with antiserum 709, H5, or H9 as indicated, after photoaffinity labeling in the absence (−) or presence (+) of 1 μM MK-886. The migration positions of $^{14}$C-labeled molecular weight markers are indicated.

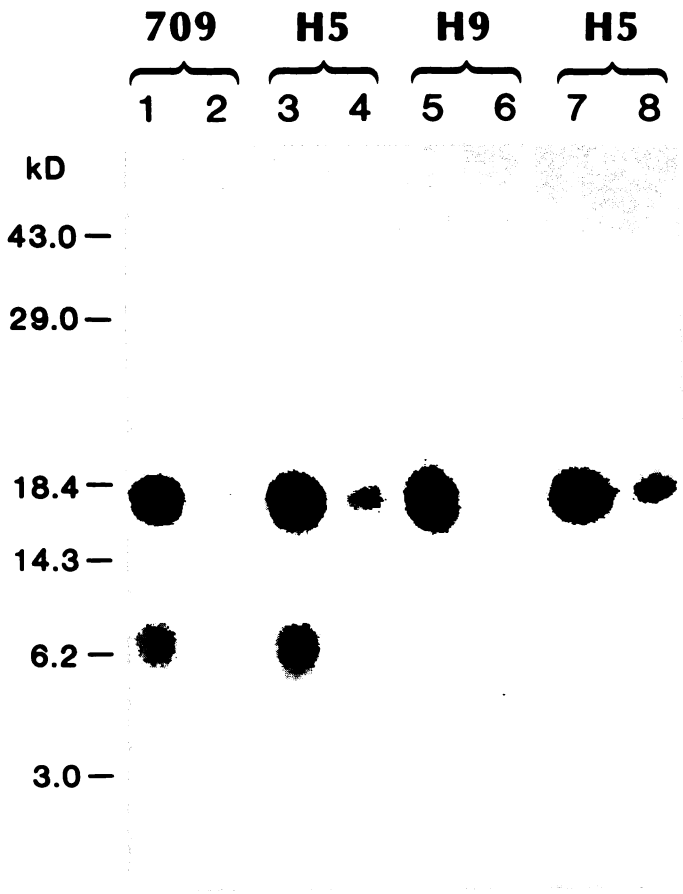


**Fig. 3.** Immunoprecipitation of photoaffinity-labeled FLAP after digestion with CNBr. Membrane preparations from human leukocytes were labeled with [$^{125}$I]L-669,083 in the absence (*lanes 1, 3, 5,* and *7*) or presence (*lanes 2, 4, 6,* and *8*) of 1 μM MK-886, as described in Materials and Methods. Samples were then incubated in the absence (*lanes 7* and *8*) or presence (*lanes 1–6*) of CNBr. After incubation, samples were immunoprecipitated with the indicated antiserum and separated by 17% SDS-PAGE. Gels were fixed, dried, and exposed to Kodak XAR-2 film for 4 days at −70°. The migration positions of $^{14}$C-labeled molecular weight markers are indicated.

associated with both undigested FLAP and the CNBr digestion fragment was competed when photoaffinity labeling was performed in the presence of 1 μM MK-886 (Fig. 3), confirming that the digestion fragment is derived from FLAP. Because 709 and H5, but not H9, recognize amino acid sequences amino-terminal to $^{89}$Met, these results suggest that the photoaffinity ligands are attached to FLAP amino-terminal to $^{89}$Met.

In the presence of 4 M guanidine-HCl, IBA cleaves polypeptide chains at peptide bonds carboxyl-terminal to tryptophan residues (34, 35). Because human FLAP contains a single tryptophan residue at position 72 (Fig. 1), digestion of FLAP at this residue should generate two polypeptides, corresponding to amino acid residues 1–72 and 73–161. To determine whether [$^{125}$I]L-669,083 and [$^{125}$I]L-691,678 attach amino- or carboxyl-terminal to $^{72}$Trp, immunoprecipitation was performed after digestion with IBA of FLAP labeled with these ligands. Fig. 4 shows that antisera 709 and H5, which recognize amino acid residues 1–39 and 41–52 of FLAP, respectively, both immunoprecipitated a photoaffinity-labeled fragment of FLAP corresponding to a molecular size of approximately 6 kDa. This fragment is of a size similar to that recognized by Western blot analysis with H5 antiserum after digestion of FLAP with IBA

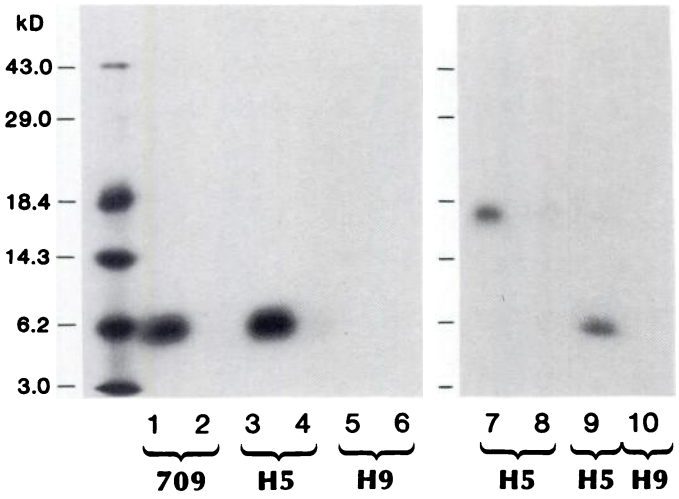


**Fig. 4.** Immunoprecipitation of photoaffinity-labeled FLAP after digestion with IBA. Membrane preparations from human leukocytes were labeled with [$^{125}$I]L-669,083 (*lanes 1–8*) or [$^{125}$I]L-691,678 (*lanes 9* and *10*) and incubated in the absence (*lanes 7* and *8*) or presence (*lanes 1–6, 9*, and *10*) of IBA, as described in Materials and Methods. Labeling was performed in the absence (*lanes 1, 3, 5, 7, 9*, and *10*) or presence (*lanes 2, 4, 6*, and *8*) of 1 $\mu$M MK-886. Samples were then immunoprecipitated with the indicated antiserum and separated by 13.5% SDS-PAGE. Gels were fixed, dried, and exposed to Kodak XAR-2 film for 3 days at −70°. The migration positions of $^{14}$C-labeled molecular weight markers are indicated.

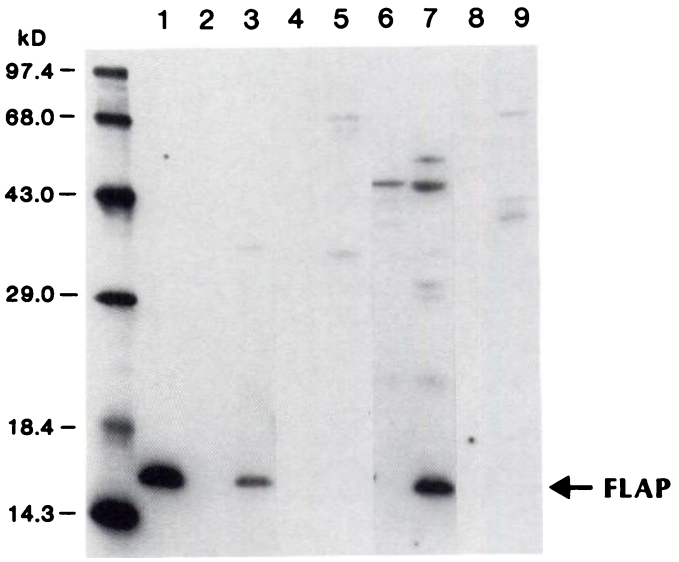


**Fig. 5.** Immunoblot analysis of FLAP. Immunoblot analysis of the 100,000 × *g* membrane fraction from human leukocytes (*lane 1*), wild-type COS-7 cells (*lanes 2, 4, 6*, and *8*), or COS-7 cells transfected with pEUKhFLAP (*lanes 3, 5, 7*, and *9*) was performed as described in Materials and Methods, using antiserum 709 (*lanes 1–3*), antiserum H5 (*lanes 6* and *7*), or preimmune sera from the rabbits used to raise antisera 709 (*lanes 4* and *5*), or H5 (*lanes 8* and *9*). Blots were exposed to Kodak XAR-2 film at −70° for 24 hr. The migration positions of molecular weight markers are indicated.

(data not shown) and is of slightly lower molecular weight than the fragment immunoprecipitated by antisera 709 and H5 after digestion of FLAP with CNBr (Fig. 3). Photoaffinity labeling of this peptide fragment was inhibited by MK-886, confirming that it is derived from FLAP. In contrast, H9 antiserum, which recognizes amino acid residues 101–118, does not immunoprecipitate a photoaffinity-labeled polypeptide after digestion of FLAP with IBA. These results suggest that the labeled fragment recognized by antisera 709 and H5 corresponds to amino acids 1–72 of FLAP and are consistent with the studies using CNBr (Fig. 3), which demonstrated that the photoaffinity ligands are attached to FLAP amino-terminal to $^{89}$Met.

The immunoprecipitation studies described above localize the site of attachment on FLAP for photoaffinity analogues of leukotriene biosynthesis inhibitors to a region of the protein that includes the hydrophilic loop between the first and second transmembrane regions. These findings provided a focus for site-directed mutagenesis studies to define more accurately the amino acid residues of FLAP critical for the binding of inhibitors. By immunoblot analysis using antisera 709 and H5 (Fig. 5), there was no detectable expression of FLAP in wild-type COS-7 cells. This finding is consistent with the inability of this monkey kidney cell line to synthesize leukotrienes in response to challenge with the calcium ionophore A23187.[2] However, FLAP was expressed and localized to the 100,000 × *g* membrane fraction of these cells after transfection with the expression vector pEUKhFLAP (Fig. 5). FLAP was undetectable in the 100,000 × *g* supernatant fraction of the cells after transfection with pEUKhFLAP (data not shown), demonstrating that FLAP is effectively localized to the membrane after expression in these cells.

In order to assess the significance of altering amino acid residues of FLAP on the binding of leukotriene biosynthesis inhibitors, it was first necessary to assess whether hFLAP expressed in COS-7 cells has binding characteristics similar to those exhibited by FLAP from human leukocytes. A recently described radioligand binding assay utilizing the quindole [$^{125}$I]L-691,831 was used for these studies (22). Consistent with immunoblot analyses (Fig. 5), no specific binding of [$^{125}$I]L-691,831 was detectable in membrane preparations from wild-type COS-7 cells (data not shown). Full saturation analysis in the radioligand binding assay of membrane preparations from COS-7 cells transfected with pEUKhFLAP (Fig. 6) demonstrates that specific binding of [$^{125}$I]L-691,831 to FLAP increased with increasing concentration of radioligand. Scatchard analysis of this saturation curve (Fig. 6) is consistent with a one-site model for specific binding, with a dissociation constant for [$^{125}$I]L-691,831 of approximately 5 nM. The $B_{max}$ determined by this analysis varied between 1.4 and 3.5 pmol/mg of protein in different experiments, due to differences in the levels of FLAP achieved in different transfections. These levels of FLAP are 4–9-fold less than those reported in membrane preparations from human leukocytes (22). Scatchard analysis of [$^{125}$I]L-691,831 binding to FLAP from human leukocytes is also consistent with a single ligand binding site, with a dissociation constant for [$^{125}$I]L-691,831 of approximately 6 nM (22). Full competition analysis of membrane preparations from human leukocytes or COS-7 cells transfected with pEUKhFLAP were performed in the presence of MK-886, L-689,037, or REV-5901. These compounds are members of the indole, quindole, and quinoline series of leukotriene biosynthesis inhibitors, respectively, and have $IC_{50}$ values for the inhibition of leukotriene synthesis in hPMN of 3 nM, 2 nM, and 1.8 $\mu$M (21, 22). Fig. 7 shows that FLAP expressed in COS-7 cells (Fig. 7A) and present in human leukocytes (Fig. 7B) displayed a similar affinity for these three inhibitors, consistent with the Scatchard

[2] J. A. Mancini and P. J. Vickers, unpublished observations.

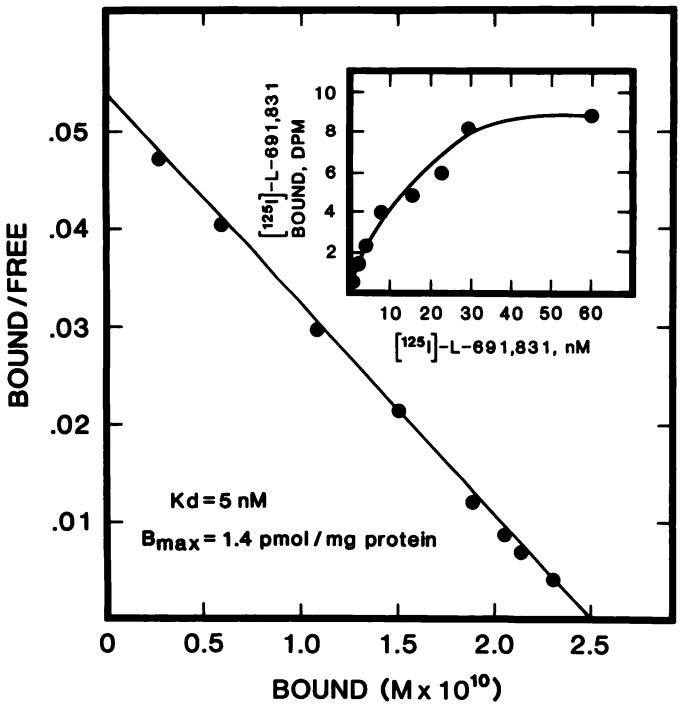


**Fig. 6.** Scatchard analysis of [$^{125}$I]L-691,831 binding to membrane preparations from COS-7 cells. COS-7 cells were transfected with the expression vector pEUKhFLAP, and the 100,000 × *g* membrane fraction was prepared as described in Materials and Methods. Saturation analyses of membrane preparations (*inset*) were performed in duplicate with [$^{125}$I]L-691,831, as described in Materials and Methods, using the indicated concentrations of radioligand. Scatchard analysis of this saturation curve was performed using nonlinear regression analysis of a one-site fit model, using LIGAND software, according to the method of Munson and Rodbard (29).

analysis (Fig. 6), which suggested that FLAP expressed in COS-7 cells has essentially the same binding characteristics as FLAP from human leukocytes.

A series of deletion and point mutants of human FLAP focused in the first hydrophilic loop of FLAP were developed to determine whether amino acids in this region of the protein are essential for the binding of leukotriene biosynthesis inhibitors. Fig. 8 shows a typical immunoblot analysis, using 709 and H5 antisera, of membrane preparations from COS-7 cells that were transfected with expression vectors for various FLAP mutants. By this analysis, all of the mutants that contain alterations in the first hydrophilic loop of FLAP were expressed to similar levels and localized to the membrane fraction of COS-7 cells. Whereas mutants in which the peptide sequence recognized by a peptide antiserum was disrupted could not be detected by that antiserum, they were detected by the alternate antiserum [e.g., hFLAP(del 37–53) was detectable by 709 but not H5 antiserum] (Fig. 8). FLAP mutants could not be detected in cytosol or membrane fractions of COS-7 cells that were transfected with cDNA constructs in which either one or more transmembrane regions of FLAP were deleted or a number of amino acids proposed to be in a transmembrane region were deleted [e.g. hFLAP(del 63–67) or hFLAP(del 68–71)]. We are currently attempting to express and analyze mutants of FLAP containing shorter deletions in transmembrane regions of the protein.

The initial step in determining the ability of FLAP mutants to bind [$^{125}$I]L-691,831 was to measure the specific binding displayed by membrane preparations containing these mutants, as previously described (22). By this analysis, hFLAP(del 33–36), hFLAP(del 37–41), and hFLAP($^{62}$Asp-Glu) displayed levels of specific binding similar to those of hFLAP (approximately 500 fmol/mg of protein). For these mutants, specific binding was linear with increasing protein concentration (data not shown). To quantitate accurately the effects of these mutations upon the affinity of FLAP for leukotriene biosynthesis inhibitors, membrane preparations from COS-7 cells were analyzed by full competition analysis in the radioligand binding assay, using the quindole L-689,037 as the competing ligand. Fig. 9 shows that, for hFLAP(del 33–36), hFLAP(del 37–41), and hFLAP($^{62}$Asp-Glu) mutants, specific binding of [$^{125}$I]L-691,831 to the mutant protein was competed by L-689,037 in a concentration-dependent manner, with the dissociation constant for these mutants being in a range between 3 and 10 nM. These values are similar to that of 2 nM determined for wild-type FLAP under the same conditions, suggesting that amino acid residues 33–41 in the first hydrophilic loop of FLAP do not play a critical role in the binding of [$^{125}$I]L-691,831.

For membrane preparations in which hFLAP(del 37–53),

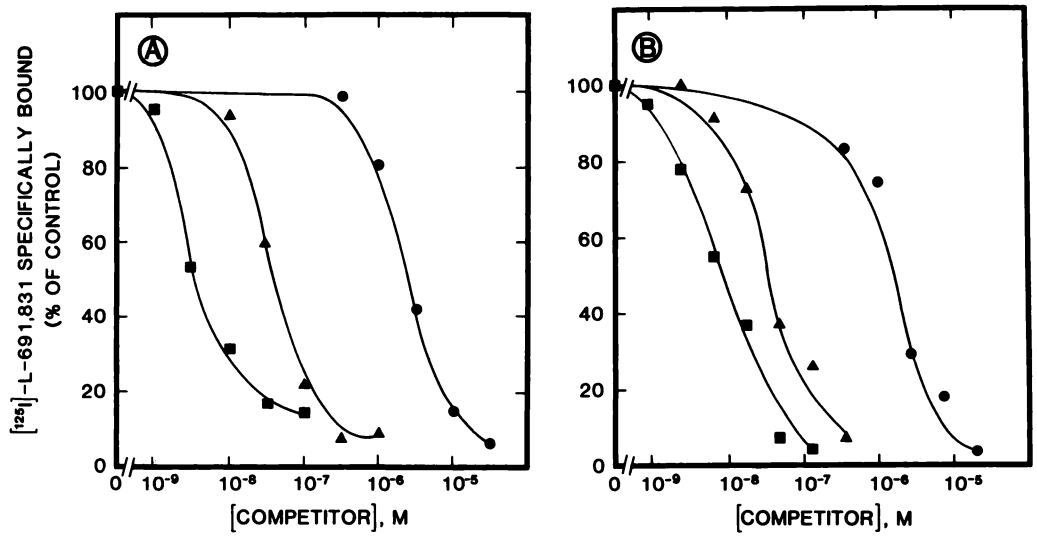


**Fig. 7.** Competition analysis of [$^{125}$I]L-691,831 binding to FLAP. The binding of [$^{125}$I]L-691,831 to 100,000 × *g* membrane preparations from COS-7 cells transfected with pEUKhFLAP (A) or human leukocytes (B) was analyzed in the presence of the indicated concentrations of L-689,037 (■), MK-886 (▲), or REV-5901 (●), as described in Materials and Methods. Values represent the mean of duplicate assays.

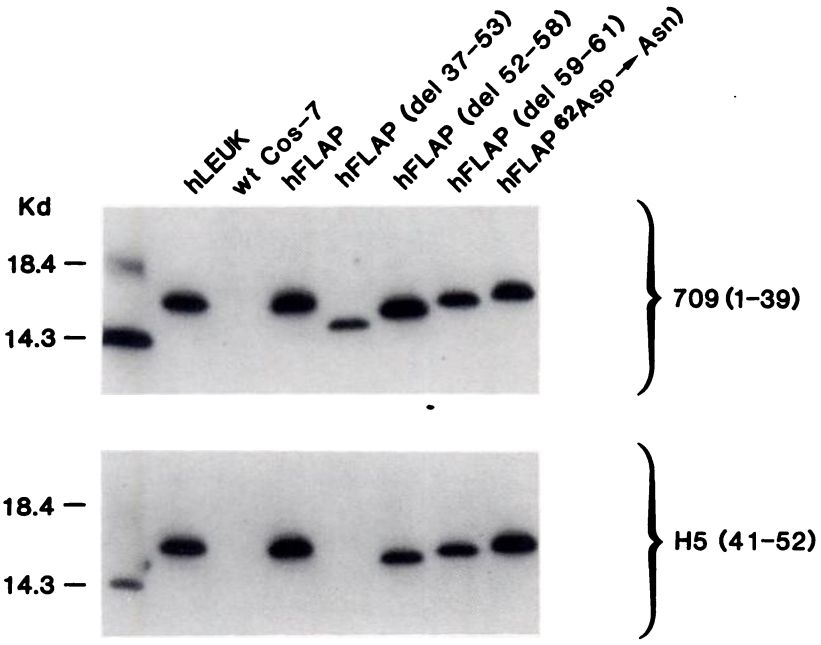


**Fig. 8.** Immunoblot analysis of FLAP mutants. Membrane preparations (100,000 × *g*) (100 μg) from human leukocytes (*hLEUK*), wild-type COS-7 cells (*wt Cos-7*), or COS-7 cells transfected with expression vectors for hFLAP, hFLAP(del 37–53), hFLAP(del 52–58), hFLAP(del 59–61), or hFLAP($^{62}$Asp-Asn) were analyzed by immunoblot, using antiserum 709 or H5, as indicated. Regions of the blots in which FLAP and FLAP mutants migrate are shown, with the migration positions of molecular weight markers indicated. The amino acid residues of FLAP recognized by antisera 709 and H5 are shown. By immunoblot analyses, the mutants hFLAP(del 33–36) and hFLAP($^{62}$Asp-Glu) were expressed at levels similar to those of the aforementioned mutants (data not shown).

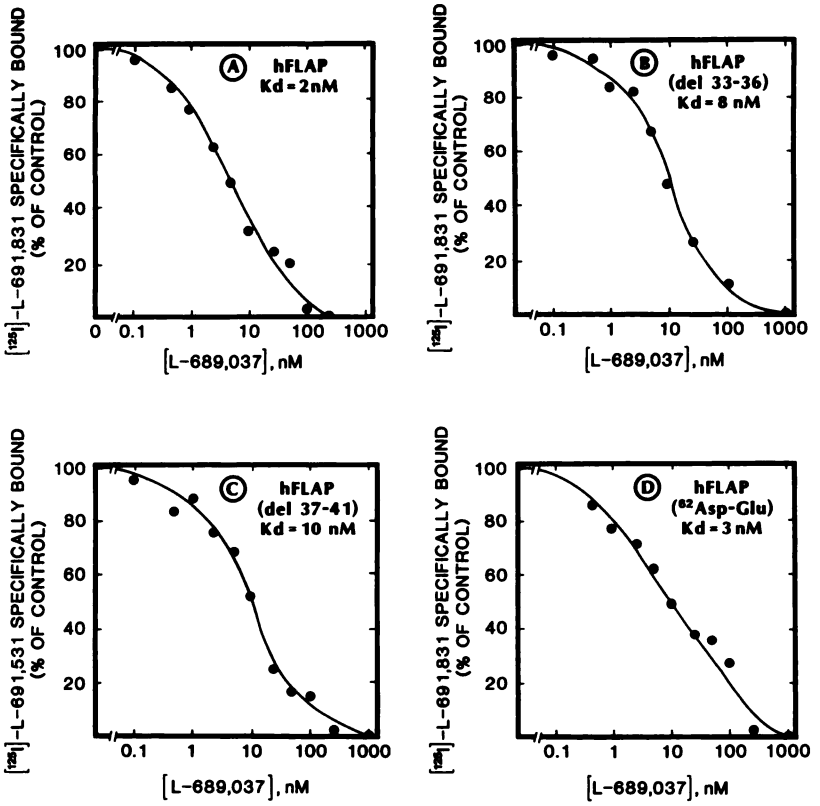


**Fig. 9.** Competition analysis of [$^{125}$I]L-691,831 binding to FLAP mutants. The binding of [$^{125}$I]L-691,831 to 100,000 × *g* membrane preparations from COS-7 cells transfected with expression vectors for hFLAP (A), hFLAP(del 33–36) (B), hFLAP(del 37–41) (C), or hFLAP($^{62}$Asp-Glu) (D) was analyzed in the presence of the indicated concentrations of L-689,037, as described in Materials and Methods. The indicated dissociation constant ($K_d$) values were determined using the LIGAND program of Munson and Rodbard (29).

hFLAP(del 52–58), or hFLAP(del 59–61) was present, specific binding of [$^{125}$I]L-691,831 was undetectable up to protein concentrations of 30 μg of protein/assay. For hFLAP($^{62}$Asp-Asn), very low levels of specific binding (approximately 20 fmol/mg of protein) could be detected at high protein concentrations (20–80 μg of protein/assay). This low level of specific binding was insufficient to perform accurate quantitative analysis. These results demonstrate that a number of residues in the carboxyl-terminal half of the proposed first hydrophilic loop of FLAP and a negative charge associated with amino acid residue 62 are critical for inhibitor binding.

## Discussion

We have used two approaches to determine amino acid residues of human FLAP involved in the binding of leukotriene biosynthesis inhibitors. Antipeptide antisera that recognize three different regions of human FLAP immunoprecipitate a single radiolabeled species after photoaffinity labeling of the protein with radiolabeled analogues of two classes of inhibitors. This finding has allowed a determination of the peptide fragments to which these ligands are specifically attached after digestion of the labeled protein with reagents that specifically cleave FLAP at methionine or tryptophan residues. These

studies demonstrate that the photoaffinity ligands are attached to FLAP amino-terminal to $^{72}$Trp.

In order to extend the photoaffinity labeling studies, we used COS-7 cells to express FLAP and site-directed mutants of FLAP for analysis in a radioligand binding assay. As determined with this assay and immunoblot analysis, wild-type COS-7 cells contain no FLAP, whereas FLAP is expressed and localized to the membrane fraction in COS-7 cells transfected with the expression vector pEUKhFLAP. As assessed by Scatchard analysis and competitive radioligand binding assays with members of the indole, quinoline, and quindole classes of leukotriene biosynthesis inhibitors, FLAP expressed in COS-7 cells exhibits the same binding characteristics as the protein from human leukocytes and is, therefore, a suitable system for analyzing the binding characteristics of FLAP mutants.

Using the radioligand binding assay, analysis of membrane preparations from COS-7 cells containing the deletion mutants hFLAP(del 33–36) and hFLAP(del 37–41) demonstrates that amino acid residues at the amino terminus of the first hydrophilic loop of FLAP are not essential for the binding of leukotriene biosynthesis inhibitors. This finding is supported by a comparison of the amino acid sequences of rat FLAP and hFLAP (23). FLAP from these species are 92% identical at the amino acid level, with only 13 amino acid substitutions. However, four of these substitutions are located at amino acid residues 32, 35, 36, and 38 at the amino terminus of the first hydrophilic loop (Fig. 1). Because FLAP from either of these species specifically binds leukotriene biosynthesis inhibitors, these four amino acids cannot play a critical role in inhibitor binding. In contrast to results with hFLAP(del 33–36) and hFLAP(del 37–41), mutants in which parts of the carboxyl terminus of the first hydrophilic loop were deleted [hFLAP(del 37–53), hFLAP(del 52–58), and hFLAP(del 59–61)] lost all ability to bind specifically the leukotriene biosynthesis inhibitor [$^{125}$I]L-691,831. Taken together, these results demonstrate that, within the region from $^{41}$Ser to $^{61}$Val in the first hydrophilic loop of human FLAP, a number of amino acid residues are critical for the binding of leukotriene biosynthesis inhibitors. Furthermore, a point mutation of $^{62}$Asp to an asparagine residue, resulting in the loss of the negative charge associated with this residue, results in the generation of a mutant protein that can bind only very low levels of [$^{125}$I]L-691,831. If the negative charge associated with residue 62 is retained by conversion to a glutamate residue, the resulting mutant exhibits essentially the same binding characteristics as the wild-type protein. The specific roles that amino acid residues at the carboxyl terminus of the first hydrophilic loop of FLAP play in inhibitor binding, and whether amino acid residues located elsewhere in the protein are involved in forming the inhibitor binding site, are currently under investigation.

Analysis of the amino acid sequence in the first hydrophilic loop of FLAP suggests that it may form an amphipathic $\alpha$-helix, with the charged residues $^{40}$Arg, $^{44}$Arg, $^{51}$Glu, and $^{52}$Arg oriented on the same side of the helix (data not shown). This structural feature occurs in a number of proteins and peptides involved in protein-protein and peptide-protein interactions (36–38). Because mutants of FLAP containing amino acid deletions between amino acids 41 and 61 have lost the ability to bind specifically leukotriene biosynthesis inhibitors and these inhibitors specifically block the membrane association of 5-LO, this amphipathic helix may be considered a potential interaction site between FLAP and either 5-LO or a phospholipase involved in leukotriene synthesis. We are currently attempting to express deletion mutants of FLAP with 5-LO, to assess whether the same amino acids that are critical for the binding of leukotriene biosynthesis inhibitors are also critical for supporting leukotriene synthesis and the membrane translocation of 5-LO. The identification in these studies of amino acid residues of FLAP that are essential for the binding of leukotriene biosynthesis inhibitors may, therefore, lead to studies that clarify both the role FLAP plays in leukotriene biosynthesis and the mechanism by which compounds that specifically bind to FLAP inhibit this process.

**Acknowledgments**

The authors would like to thank R. Dixon and R. Diehl for providing the cDNA for human FLAP, P. Charleson and J.-M. Dufour for the radioiodination of [$^{125}$I]L-691,678 and [$^{125}$I]L-691,831, D. K. Miller and E. Rands for providing antiserum 709, T. Nguyen and D. K. Miller for helpful discussions, and B. Sholzberg for secretarial services.

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