[Frontiers in Bioscience 2, d380-386, July 15, 1997]

LEUKOTRIENE C_4 SYNTHASE: A CRITICAL ENZYME FOR THE BIOSYNTHESIS OF SRS-A.

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1. ABSTRACT

Leukotriene (LT) C_4 synthase catalyzes the conjugation of LTA_4 with reduced glutathione (GSH) to form LTC_4, the parent compound of cysteinyl leukotrienes. It is a 18 kDa protein that functions as homodimer. Cloning of LTC_4 synthase cDNA reveals amino acid homology with 5-lipoxygenase activating protein (FLAP) and newly identified microsomal glutathione S-transferase II (mGST-II) but not with cytosolic GSTs or mGST-I. LTC_4 synthase gene contains 5 exons and four introns. This gene has been localized to the long arm of human chromosome 5 at the region of 5q35 which is in close proximity to the cluster of genes that are involved in inflammation and asthma. Mutagenic studies reveals that amino acid residues Arg-51 and Tyr-93 are critical for catalytic function. Arg-51 was proposed to open the epoxide ring of LTA_4 and Tyr-93 to provide the thiolate anion of GSH.

2. INTRODUCTION

Asthma is characterized by episodic bronchoconstriction, hyper-responsiveness, and inflammation of the conducting airways (1,2). The inflammatory responses in asthma include luminal plugging with mucus, epithelial cells, and eosinophils (3,4); denudation of the epithelium (5) with subepithelial collagen deposition (6); and infiltration of the epithelium and submucosa with eosinophils and TH-2 cells (7-9). In addition, there are partial degranulation of mast cells and eosinophils (7) and release of both the preformed granule enzymes and the newly-generated lipid mediators such as platelet activating factor and leukotriene (LT) C_4. These bioactive molecules may elicit many of the pathologic changes seen in asthma (2).

The specific involvement of leukotrienes in asthma is suggested by their potent biological activities, their presence in the airways in asthma, and the effect of 5-lipoxygenase (5-LO) inhibitors and LTD_4 receptor antagonist in asthma. Inhaled LTC_4 or LTD_4 are 1,000-fold, and LTE_4 10-fold, more potent than histamine in compromising airway function in normal subjects. LTC_4, LTD_4 and LTE_4 displayed similar potency and are more active in reducing lung functions in patients with asthma (10). They stimulate mucus secretion from bronchial epithelial cells and increase pulmonary vascular permeability via endothelial cell contraction at the post capillary venules (11-13). The cysteinyl leukotrienes have been recovered from the airways of asthmatic individuals both at rest (14) and following allergen challenge (15) or isocapnic hyperventilation (16). Clinical studies with 5-LO inhibitors and 5-lipoxygenase activating protein (FLAP) inhibitors, agents that inhibit leukotrienes formation, and with leukotriene antagonists, has permitted a definitive examination of the role of leukotrienes in bronchial asthma. Administration of these agents, which devoid of intrinsic bronchodilatory activity, leads to bronchodilatation in patients with asthma (17,18), suggesting that leukotrienes contribute to the abnormal resting airway tone in asthma. These compound inhibit acute asthmatic response to exercise (19,20), cold-dry air (21), allergen (22) and aspirin (23,24). They decrease the late asthmatic response to allergen and the accompanying airway hyper-responsiveness (22), and reduced the severity of chronic asthma (25), all of which support the role of cysteinyl leukotrienes contributing to the pathogenesis of bronchial asthma.

Received 7/11/97 Accepted 7/15/97
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M K D E V A L L A A V T L L G V L L Q A Y F S L *Q*
V I ***S A R*** R A F R V S P P L T T G P P E F E R V Y 50

R A Q V N C S E Y F P L F L A T L W V A G I F F H
E G A A A L C G L V Y L F A R L R ***Y*** F Q G *Y* A R S 100

A Q L R L A P L *Y* A ***S A R*** A L W L L V A L A A L G
L L A H F L P A A L R A A L L G R L R T L L P W A 150

Figure 1. Deduced amino acid sequence of human LTC_4 synthase. Three hydrophobic domains are underlined. Two putative protein kinase C phosphorylation sites are shown in bold and italic. Residues Arg-51 and Tyr-93, which effect the catalytic function of LTC_4 synthase, are shown in a larger font. Amino acid residue numbers are on the right.

The formation of cysteinyl leukotrienes in mast cells and eosinophils is initiated upon agonist stimulation and increase in intracellular Ca^{++} and activation of cytosolic PLA_2. PLA_2 hydrolyze and release arachidonic acid (AA) from membrane phospholipids (26). The released AA binds to 5-lipoxygenase activating protein (FLAP) and is presented to 5-lipoxygenase (27). 5-lipoxygenase converts AA to 5-hydroperoxy-eicosatetraenoic acid (5-HPETE) and subsequently to LTA_4 (28). LTC_4 synthase, an integral membrane protein, conjugates LTA_4 with reduced glutathione (GSH) to form the LTC_4 (29,30), the parent compound of cysteinyl leukotrienes. Following carrier-mediated export (31), sequential cleavage of glutamic acid and glycine from the glutathione moiety of LTC_4 yields the receptor active derivatives, LTD_4 and LTE_4 (32,33), respectively.

3. BIOCHEMICAL PROPERTIES OF NATIVE LTC_4 SYNTHASE

Although LTC_4 synthase conjugates glutathione, it was recognized to differ other glutathione S-transferase (GST), by its selectivity for LTA_4 and its analogs, and failure to conjugate xenobiotics (34). The enzyme is expressed in a limited number of cell types such as eosinophils (35), mast cells (36) and monocyte/macrophages (37) and in leukemic cell lines such as KG-1 cells (38) and THP-1 cells (39); it is also present in platelets (40) which lack 5-lipoxygenase.

Certain cytosolic and microsomal GSTs can conjugate GSH to either a xenobiotic or LTA_4 (41,42), but their role in the inflammatory response is not evident at present. LTC_4 synthase from guinea pig lung, KG-1, THP-1 and U-937 cells as well as from human platelets (29,38-39,43-44) has been distinguished from cytosolic and microsomal GSTs not only by a narrow substrate specificity, but also by differential susceptibility to inhibitors and immunoreactivity. Partially purified LTC_4 synthase isolated from guinea lung has been demonstrated to conjugates only LTA_4 and its structural isomers with GSH and does not utilize xenobiotics as substrates (Table 1) (29,30). LTC_4 synthase purified from the DMSO-differentiated U-937 cells (>10,000 fold purification) displayed K_m values for LTA_4 and GSH of 19.6 μM and 1.83 mM, respectively, in the same range as the partially purified guinea pig enzyme but with a V_{max} value of 2-4 μmol/min/mg (43). LTC_4 synthase activity is augmented by Mg^{++} ions and phosphatidylcholine, and is inhibited by Co^{++} ions. N-ethylmaleimide, an agent which activates microsomal GSTs, inhibits LTC_4 synthase both from guinea pig lung and from U-937 cells (29,43).

Table I. Substrate specificity of LTC_4 synthase from guinea pig lung.*

Substrate	K_m	V_{max}
LTA_3	4.3 μM	16 ηmol/min/mg
LTA_4	3 μM	36 ηmol/min/mg
LTA_5	2.6 μM	18 ηmol/min/mg
LTA_4-methylester	16 μM	140 ηmol/min/mg
14,15-LTA_4	77 μM	68 ηmol/min/mg
5-epi-LTA_4	38 μM	31 ηmol/min/mg
6-epi-LTA_4	24 μM	54 ηmol/min/mg
5-epi,6-epi-LTA_4	27 μM	34 ηmol/min/mg
GSH	2.3 mM	-----

* Data were obtained from Yoshimoto *et al*, 1988 (29).

LTC_4 synthase isolated from THP-1 cells and from DMSO-differentiated U-937 cells does not cross react with antibodies to human microsomal GST or to human alpha, Mu and Pi class of cytosolic GSTs (39,43). In contrast, rabbit polyclonal antiserum raised against purified human lung LTC_4 synthase recognized the 18 kDa LTC_4 synthase purified from KG-1 cells, human lung, and COS cells transfected with LTC_4 synthase cDNA in western blot analysis (45). In addition, this antibody also recognized an 18 kDa protein from human platelets, *in vitro* derived human eosinophils and mouse bone marrow-derived mast cells (45,46). Immunohistochemical analysis of sections of human lung disclosed that the subcellular localization of human LTC_4 synthase in alveolar macrophages is perinuclear in distribution (45).

Recently, LTC_4 synthase was purified to homogeneity as an 18 kDa protein from human leukemic KG-1 cells using probenecid as a novel elution reagent for S-hexyl glutathione chromatography followed by gel electrophoresis (38). Purification of an 18 kDa protein was also achieved from THP-1 cells (39). The 18 kDa protein was proposed to function as a homodimer based on the size of the active fraction by gel filtration column chromatography (39,47).

4. LTC_4 SYNTHASE cDNA

A novel fluorescence-linked competitive immunoassay, sensitive to 2.5 pg of LTC_4, was developed for expression cloning of human LTC_4 synthase (48). Using this method for first round of screening of 2.5×10^5

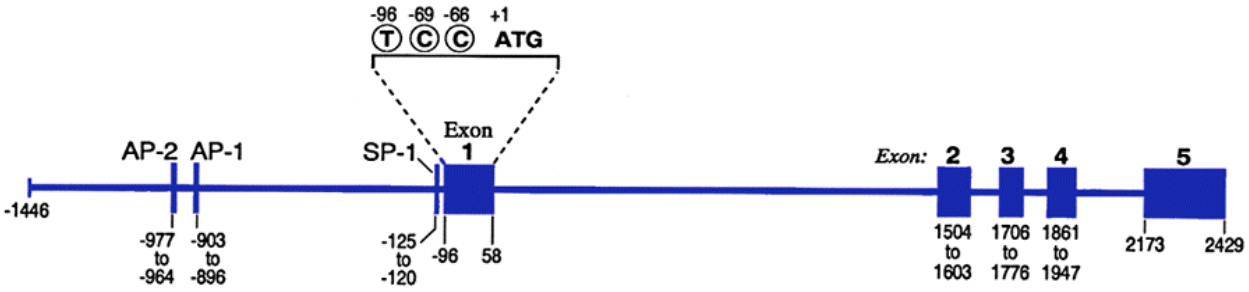

Figure 2. Schematic representation of the genomic organization of the human LTC4 synthase gene. Nucleotide numbers start with the translation ATG codon. Putative transcription initiation sites are circled. Exons and the putative transcription regulation elements are boxed. Modified from Penrose *et al*, 1996 (51).

colonies of a pcDNA3 expression library and subsequent round of screening by RP-HPLC, we were able to isolat the cDNA clone for human LTC_4 synthase. The positive clone contained a 694 bp cDNA insert with a 450 bp open reading frame. It encodes for a 150 amino acid residue polypeptide (Figure 1)(48). The deduced amino acid sequence of LTC_4 synthase reveals 2 potential protein kinase C (PKC) phosphorylation sites and 1 potential N-glycosylation site.

The nucleotide sequence of the cDNA and the deduced amino acid sequence of human LTC_4 synthase do not share significant homology with the xenobiotic active GSTs. However, protein sequence analysis revealed 31% overall amino acid identity with FLAP. The amino acid identity at the N-terminal 2/3 of these proteins is 44% which includes the putative FLAP inhibitor binding domain (Fig. 1)(49). The predicted secondary structure of the deduced LTC_4 synthase is nearly identical to that of FLAP with their 3 hydrophobic domains overlap each other and 2 identical sized hydrophilic loops.

These features of human LTC4 synthase was conserved in mouse LTC4 synthase (50). Mouse LTC4 synthase is also an 150 amino acid residue polypeptide. It differs from human LTC_4 synthase in 18 amino acid residues, 9 of which are located at the carboxy-terminus of the protein. The putative FLAP inhibitor binding domain (with 2 conservative substitution of Y50F and T41S), the potential N-glycosylation site and the two potential PKC phosphorylation sites are present in mouse LTC_4 synthase.

Recently, a new microsomal glutathione S-transferase (mGST-II) has been identified that displayed 44% overall amino acid identity with human LTC_4 synthase (42) which also includes the putative FLAP inhibitor binding domain-like region. The predicted secondary structure of mGST-II is similar to that of LTC_4 synthase and FLAP suggesting that these three proteins belongs to a new gene family.

5. BIOCHEMICAL PROPERTIES OF RECOMBINANT LTC_4 SYNTHASE

Kinetic analysis of S-hexyl-GSH agarose affinity purified recombinant human and mouse LTC_4 synthase showed near identical K_m and V_{max} values (Table 2). These K_m and V_{max} values are also in close agreement with the published value for LTA_4 free acid and GSH using enzyme purified from human leukemic cells (43). The amino acid residue substitutions in the putative FLAP inhibitor binding domain-like region of mouse LTC_4 synthase did not modify the effect of a FLAP inhibitor, MK-886. The IC_{50} for human and mouse recombinant LTC_4 synthase were 3.1 μM and 2.7 μM, respectively. These results indicate that the amino acid substitutions observed in mouse LTC_4 synthase do not affect enzyme function and further suggest that the carboxyl terminal end of LTC_4 synthase are not important in either the binding or the catalytic function of the enzyme.

6. CATALYTIC MECHANISM OF CONJUGATION

Site-directed mutagenesis was carried out to determine the substrate binding site and the catalytic mechanism of human LTC_4 synthase (47). Mutation of the Arg-51 of the first hydrophilic loop to Thr or Ile abolishes function of the recombinant protein, whereas mutations of Arg-51 to His or Lys provide a fully active recombinant protein. Mutation of V49F, A52S, N55A and Y59F of the first hydrophilic loop, and mutations of Y97F and Y93F of the second hydrophilic loop, increases the K_m of the recombinant enzyme for GSH suggested that these residues are involved in GSH binding. The mutation of Y93F also markedly reduces function and shifts the optimum for pH dependent activity. Direct linkage of two LTC_4 synthase monomers by a 12 amino acid bridge provides an active covalent-linked dimer and identical bridging of inactive R51I with a wild type monomer also creates an active pseudo-heterodimer. These results suggest that Arg-51 and Tyr-93 are involved in catalysis with Arg-51 opening the epoxide ring and Tyr-93 providing the thiolate anion of glutathione. That a wild type monomer linked to an inactive monomer has full function suggests that each monomer of LTC_4 synthase has independent conjugation activity and that dimerization of LTC_4 synthase maintains the proper protein folding. It is interesting to point out that the amino acid residue Tyr-93 is present in both mGST-II and FLAP. In contrast, Arg-51 is present in mGST-II, which has the ability to conjugate

Table II. Kinetic parameters of purified recombinant mouse and human LTC_4 synthase

	Mouse LTC_4 Synthase		Human LTC_4 Synthase	
Substrates/Inhibitor	**K_m/IC_{50}**	**V_{max}**	**K_m/IC_{50}**	**V_{max}**
LTA_4-methylester	10.3 μM	2.3 μmol/min/mg	7.6 μM	2.5 μmol/min/mg
LTA_4	2.5 μM	1.2 μmol/min/mg	3.6 μM	1.3 μmol/min/mg
GSH (LTA_4-methylester)	1.9 mM	2.2 μmol/min/mg	1.6 mM	2.7 μmol/min/mg
MK-886(IC_{50})	3.1 μM		2.7 μM	

Data were obtained from Lam *et al*, 1996 (50)

both LTA_4 and xenobiotics with GSH, but is not present in FLAP which has no known GSH conjugating activity.

7. GENOMIC ORGANIZATION OF HUMAN LTC4 SYNTHASE GENE

Human LTC_4 synthase gene consists of 5 exons and 4 introns that span 2.51 kbp. (51). The exons (ranging from 71 to 257 nucleotides) of LTC_4 synthase gene are identical in size to those of FLAP with the exception of the first and fifth, which are affected minimally by the number of nucleotides in the 5' and 3' untranslated regions. Furthermore, these exons of LTC_4 synthase and FLAP align identically with regard to the predicted secondary structures and the amino acids that they encode (48). In contrast to the exons, the introns of LTC_4 synthase are only 1/10th that of FLAP.

5’ extension analysis of KG-1 mRNA revealed three putative transcription initiation sites in the human LTC_4 synthase gene, located 66, 69, and 96 nucleotides upstream of the ATG translation start site. Similar analysis using total RNA reveals single transcription start site in THP-1 cells (52). The 1.4 kbp of the 5’ flanking region of human LTC_4 synthase gene contains SP-1, AP-1 and AP-2 consensus binding sequences (Figure 2). The presence of AP-1 and AP-2 regulatory elements are consistent with the induction of LTC_4 synthase activity by PMA (53). The FLAP gene, however, has a single transcription start site and a modified TATA box (54).

8. CHROMOSOMAL LOCALIZATION OF HUMAN LTC_4 SYNTHASE GENE

Fluorescent *in-situ* hybridization localized LTC_4 synthase gene to the long arm of chromosome 5 at an area that corresponds to band 5q35 (51). Human FLAP gene, however, was localized to 13q12. The region proximal of 5q35 has recently been identified as the site at which many of the genes encoding growth factors, cytokines, and receptors relevant to the asthmatic phenotype are localized, these include IL-3, IL-4, IL-5 and GM-CSF (55). The inflammatory response in bronchial asthma is focussed on the activation of TH2 cells, eosinophils, and mast cells. IL-4 is essential to the dominance of the TH2 phenotype and to the cell switch required for IgE biosynthesis. IL-4 and IL-5 are involved in eosinophil infiltration, and IL-3, IL-5 and GM-CSF enhance the survival and function of eosinophils, including generation of LTC_4. Furthermore, the disproportionate expression of certain genes within these regions of human chromosome 5 have been shown by genetic linkage studies to be highly correlated with atopic asthma.

9. PERSPECTIVE

Major advances have been made in the past 10 years since the initial description of SRS-A about 5 decades ago. The progressive purification and functional characterization of SRS-A led to elucidation of its composition, as cysteinyl leukotrienes. The stereochemistry of LTC_4 and its metabolites, LTD_4 and LTE_4, were subsequently defined by organic synthesis and by the demonstration that these synthetic compounds were equipotent to biologically derived materials in bioassays. The proteins involved in the biosynthesis of LTC_4, i.e. 5-LO, FLAP and LTC_4 synthase were defined and cloned. cDNA sequences and genomic organizations of these proteins revealed that two of these proteins, LTC_4 synthase and FLAP, defined a new gene family. The amino acid residues responsible for the function of each of the three proteins have recently been defined and attention is now being directed to regulation of the genes and/or their polymorphism and their implications for airway diseases. Most importantly, the cysteinyl leukotrienes contained in SRS-A have proven to be exquisitely potent in impairing airflow of human airway through its contractile activity on bronchial and vascular smooth muscle. Agents that inhibit the biosynthesis cysteinyl leukotrienes or block the receptor-mediated action are, therefore, therapeutically effective in treating patients with bronchial asthma.

10. ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health Grants AI22531, AI31599, ES06105, HL03208, and HL36110.

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