Molecular Cloning of the Gene for Human Leukotriene C₄ Synthase

ORGANIZATION, NUCLEOTIDE SEQUENCE, AND CHROMOSOMAL LOCALIZATION TO 5q35*

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Leukotriene C₄ (LTC₄) synthase catalyzes the conjugation of LTA₄ with reduced GSH to form LTC₄, the parent of the receptor active cysteiny1 leukotrienes implicated in the pathobiology of bronchial asthma. Previous cloning of the cDNA for human LTC₄ synthase demonstrated significant homology of its amino acid sequence to that of 5-lipoxygenase activating protein (FLAP) but none to that of the GSH S-transferase superfamily. Genomic cloning from a P1 library now reveals that the gene for LTC₄ synthase contains five exons (ranging from 71 to 257 nucleotides in length) and four introns, which in total span 2.52 kilobase pairs in length. The intron/exon junctions of LTC₄ synthase align identically with those of FLAP; however, the small size of the LTC₄ synthase gene contrasts with the >31-kilobase pair size reported for FLAP. Confirmation of the LTC₄ synthase gene size to ensure that no deletions had occurred during the cloning was obtained by two overlapping polymerase chain reactions from genomic DNA, which provided products of the predicted sizes. Primer extension analysis with poly(A)* RNA from culture-derived human eosinophilic granulocytes or the KG-1 myelogenous cell line revealed multiple transcriptional start sites with prominent signals at 66, 69, and 96 base pairs 5' of the ATG translation start site. The 5'-flanking region revealed a GC-rich promoter sequence consistent with an SP-1 site and consensus sequences for AP-1 and AP-2 enhancer elements. 24, 807, and 877 bp, respectively, 5' from the first transcription initiation site. Southern blot analysis of a genomic DNA (with full-length cDNA as well as 5' and 3' oligonucleotide probes) confirmed the size of the gene and indicated a single copy gene in normal human genomic DNA. Fluorescent in situ hybridization mapped LTC₄ synthase to chromosomal location 5q35, which is in close proximity to the cluster of genes for cytokines and receptors involved in the regulation of cells central to allergic inflammation and implicated in bronchial asthma.

Leukotriene C₄ (LTC₄) and its active metabolites, LTD₄ and LTE₄, are the major components of the biologic activity previously known as the slow reacting substance of anaphylaxis. When inhaled, these arachidonic acid-derived lipid mediators exert profound smooth muscle constrictor effects on the airways of individuals with and without asthma (1, 2). The cysteiny1 leukotrienes are further implicated in the pathogenesis of asthma by the presence of their metabolites in the urine of patients with acute severe asthma (3). Moreover, cysteiny1 leukotriene synthesis inhibitors or receptor antagonists significantly ameliorate the persistent pulmonary function abnormalities of individuals with asthma (4) and the exacerbations of bronchial asthma elicited by exercise (5), inhalation of specific allergens (6), and the idiosyncratic response to aspirin (7, 8).

The formation of the cysteiny1 leukotrienes is initiated by transmembrane stimuli that increase the levels of intracellular calcium, leading to the translocation of cytosolic phospholipase A₂ (9) and 5-lipoxygenase to the perinuclear membrane (10). Cytosolic phospholipase A₂ liberates arachidonic acid from phospholipids (11) for presentation to 5-lipoxygenase by 5-lipoxygenase activating protein (FLAP) (12, 13), an integral perinuclear membrane protein (10). 5-Lipoxygenase catalyzes the sequential formation of 5-hydroperoxycosatetraenoic acid and the unstable epoxide, LTA₄ (14, 15). LTC₄ synthase then catalyzes the conjugation of LTA₄ with reduced GSH to form intracellular LTC₄ (16, 17). LTC₄ synthase is an 18-kDa integral membrane protein, which has been localized to the perinuclear region of alveolar macrophages (18) and recognized either by enzymatic function and/or by SDS-polyacrylamide gel electrophoresis immunoblot analysis in some hematopoietic cell populations such as eosinophils, basophils, mast cells, and platelets (19–21). After carrier-mediated export of LTC₄ (22), the GSH adduct is cleaved sequentially by γ-glutamyl transpeptidase to form LTD₄ (23) and by dipeptidases to yield LTE₄ (24), both of which are biologically active metabolites.

The cDNA and consensus amino acid sequence of LTC₄ synthase bear no homology to that of any member of the GSH S-transferase family, but instead, the deduced amino acid sequence shows significant homology to the amino acid sequence of FLAP (25, 26). The predicted secondary structure of LTC₄ synthase contains three hydrophobic domains and two hydrophilic loops, which align identically with the predicted secondary structure of FLAP (25). A sequence of 22 amino acid residues at the carboxyl terminus of the first hydrophilic loop of FLAP is believed to bind the released arachidonic acid and is the site at which FLAP inhibitors act to prevent cellular 5-li-
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Experimental Procedures

Cell Culture—KG-1 cells, (American Type Culture Collection, Rockville, MD) were cultured in RPMI 1640 medium (J RH Biosciences, Lenexa, KS), supplemented with 10% fetal calf serum (Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma) at 37°C under 5% CO₂. In vitro derived eosinophilic granulocytes were cultured from fetal cord blood mononuclear cells in RPMI 1640 with 10% fetal calf serum, 5% CO₂.

Genomic Cloning of LTC₄ Synthase—A P1 genomic library was screened in the Human Genome Systems (St. Louis, MO) with a polynucleotide chain reaction (PCR) product generated from digoxigenidase-designed from the cDNA for human LTC₄ synthase. The sense digoxigenidase, 5'-CGTTGCCTGAGACCA-3', and the antisense digoxigenidase, 5'-CGGTCACTAGAACTTTAATGATAGAG-3', corresponded to nucleotides 496–518 and 622–597, respectively, of the cDNA. A positive genomic P1 clone for LTC₄ synthase was identified, and its plasmid DNA was digested with various restriction enzymes (BamHI, TaqI, Sall, HindII, EcoRI, HaelII, XbaI, Xhol, ApaI, Sadi, EaqI) (New England Biolabs, Beverly, MA). The reaction products were separated by electrophoresis in a 1% agarose gel, transferred to a nylon membrane (Millipore, Bedford, MA), and probed with a fluorescently labeled 32P-labeled full-length cDNA for LTC₄ synthase and then exposed to Kodak XAR film for 2 weeks. A genomic DNA blot from 25 human chromosomal Localization—A genomic DNA blot from 25 human

Chromosomal Localization—A genomic DNA blot from 25 human

Exon was also carried out at 41°C for 30 min. The reaction products were precipitated by the addition of 2 μg of glycogen, 7.5 μl of 4 M NH₄OAc, and 100 μl of 100% ethanol. The reaction products were resuspended in 4 μl of sample buffer, boiled for 10 min, and resolved in a 6% acrylamide, 6.7 μM urea gel at 60 watts for ~1 h. Additionally, for identification of the specific nucleotides at the transcription initiation sites, a genomic sequencing reaction was performed with the same primer extension reaction to sequence the LTC₄ synthase genomic clone according to the dideoxy chain termination method of Sanger et al. (29). These reaction products and 32P-labeled molecular weight standards were run in parallel lanes of the same gel. The gel was dried and exposed to Kodak XAR film with two intensifying screens for 5 days.

Chromosomal Localization—A genomic DNA blot from 25 human

The introns and 1.35 and 0.59 kbp of the 5'- and 3'-flanking regions, respectively, are shown in lowercase letters. The exons are depicted in uppercase letters and in large boxes. The putative SP-1 promoter site and AP-1 and AP-2 enhancer elements are shown in small boxes, the three predominant transcription initiation sites are circled, and the ATG translation initiation site is underlined.

was carried out at 41°C for 30 min. The reaction products were precipitated by the addition of 2 μg of glycogen, 7.5 μl of 4 M NH₄OAc, and 100 μl of 100% ethanol. The reaction products were resuspended in 4 μl of sample buffer, boiled for 10 min, and resolved in a 6% acrylamide, 6.7 μM urea gel at 60 watts for ~1 h. Additionally, for identification of the specific nucleotides at the transcription initiation sites, a genomic sequencing reaction was performed with the same primer extension reaction to sequence the LTC₄ synthase genomic clone according to the dideoxy chain termination method of Sanger et al. (29). These reaction products and 32P-labeled molecular weight standards were run in parallel lanes of the same gel. The gel was dried and exposed to Kodak XAR film with two intensifying screens for 5 days.

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fluorescent in situ hybridization technique for the chromosomal localization of the LTC4 synthase gene was performed by Human Genome Systems. Briefly, purified DNA of the P1 clone from which the entire genomic sequence was obtained was labeled with digoxigenin dUTP by nick translation. Labeled probe was hybridized to normal metaphase chromosomes derived from phytohemagglutinin-stimulated peripheral blood lymphocytes, and specific hybridization signals were detected by incubating the hybridized slides with fluoresceinated antidigoxigenin antibodies followed by counterstaining with propidium iodide.

RESULTS

Characterization of Genomic Clone for Human LTC4 Synthase—

A 5.5-kbp SacI-digested fragment liberated from the P1 plasmid hybridized with the full-length cDNA for LTC4 synthase and with oligonucleotide primers from the 5' end and the 3' end of the cDNA, indicating that the full-length gene was contained within this fragment. This DNA fragment was subcloned and sequenced. The entire nucleotide sequence (Fig. 1) of the five exons and four introns, which spanned 2.52 kbp, was sequenced in both directions. 1.35 kbp of the 5' flanking region and 0.59 kbp of the 3' flanking region sequence were also obtained in both directions (Fig. 1).

Intron/exon junctions were determined by nucleotide sequence comparison with the cDNA and obey the GT-AG rule (32) (Fig. 2). The exon sequence exhibits 100% identity to that of the reported cDNA (25). The size of the exons ranged from 71 to 257 bp, and that of the introns ranged from 84 to 1445 bp (Table I).

Table I

| Exon | LTC4 synthase | FLAP |
|------|---------------|------|
| Exon I | 154 | 144 |
| Exon II | 100 | 100 |
| Exon III | 71 | 71 |
| Exon IV | 82 | 82 |
| Exon V | 257 | 478 |
| Intron I | 1445 | 9.0 x 10^3 |
| Intron II | 102 | 8.4 x 10^3 |
| Intron III | 84 | 4.2 x 10^3 |
| Intron IV | 230 | 8.6 x 10^3 |

A DNA blot was prepared from human genomic DNA and from P1 plasmid DNA, each of which was digested with KpnI and SacI, and probed with the full-length cDNA for LTC4 synthase (Fig. 4). The patterns of hybridization in the P1 and the human genomic DNA were identical.

Analysis of the Transcription Initiation Sites—The transcription initiation sites were determined by primer extension of poly(A)+ RNA derived from KG-1 cells and from in vitro derived hybrid granulocytes, in comparison with parallel lanes containing the sequence of the LTC4 synthase genomic clone and molecular weight markers, respectively (Fig. 5). Both cellular sources revealed three predominant transcription initiation start sites 66, 69, and 96 nucleotides upstream from the ATG translation start site.

Chromosomal Localization of the Gene for Human LTC4 Synthase—

The cDNA for LTC4 synthase hybridized to the genomic DNA from 8 of the 26 human-rodent somatic cell hybrids. The only chromosome common to these hybrids was chromosome 5. Fluorescent in situ hybridization with the P1 plasmid clone containing the gene for LTC4 synthase was performed to confirm the chromosomal assignment and to localize the region on the chromosome. The fluorescent in situ hybridization specifically labeled the long arm of a group B chromosome (chromosome 4 or 5) (Fig. 6A). In a subsequent experiment, a probe associated with the cri-du-chat locus, previously mapped to 5q21 by Genome Systems, and the P1 clone for LTC4 synthase were simultaneously hybridized to chromosome 5 (Fig. 6B). In that experiment, 73 of a total of 80 cells in metaphase that were analyzed exhibited specific labeling for the human LTC4 synthase gene. Measurements of 10 specifically hybridized chromosomes demonstrated that the P1 clone localized at a position 98% of the distance from the centromere to the telomere of chromosome arm 5q, an area that corresponds to band 5q35.

DISCUSSION

The cloning and sequencing of the gene for human LTC4 synthase (Fig. 1) have revealed that its intron size and chromosomal location are prominently different from the gene for FLAP, which encodes the only known homologous protein. The exon sequence of the gene has 100% identity with that of the previously reported cDNA, which encodes the 18-kDa LTC4 synthase. The differences in the intron sizes and the chromosomal localization of the genes are of potential functional consequence. The exons of the LTC4 synthase gene are much shorter than the exons of the FLAP gene, suggesting that the expression of the LTC4 synthase gene is regulated in a tissue-specific manner. The localization of the LTC4 synthase gene on chromosome 5q35 is distinct from the localization of the FLAP gene on chromosome 11p13, providing evidence for the independent evolution of these genes.
second exon. PCR product 2, corresponding to nucleotides 1573–2429, overlaps PCR product 1 in the second exon and extends through exon 5.

LTC4 synthase and oligonucleotides corresponding to the 5'-flanking region that hybridized with the full-length cDNA for LTC4 synthase protein. The entire gene was contained in a 5.5-kbp fragment of genomic DNA from peripheral blood leukocytes of a normal donor.

The observation that other genes lacking TATA sequences also had multiple transcription start sites (33, 34). LTC4 synthase exhibit this pattern (36, 37). Additionally, consensus sequences for an AP-1 site (TGAGTCAG) (38), and an AP-2 site (TC-CCCCTCCC) (39) were identified 807 and 877 nucleotides 5' of the first transcription initiation site. Both of these elements are responsive to the activation of protein kinase C by phorbol 12-myristate 13-acetate and are consistent with the observation that LTC4 synthase activity is induced in HL-60 cells (40) and human erythroleukemia cells after treatment with phorbol 12-myristate 13-acetate (41). In contrast, the FLAP gene has both a transcription initiation site residing in an A residue 74 nucleotides upstream from the ATG start codon and a modified TATA box (42).

When the entire genomic sequence, exon sequence, and amino acid sequence were each analyzed for similarity to sequences in the EMBL data base with blast programs (43), the only significant protein homologies were with the family of FLAP molecules from different species. The human gene for FLAP has been cloned and found to be >31 kbp in size. The FLAP gene also contains 5 small exons but four large introns (Table I) for which sequence data are limited to the intron/exon junctions (42). The exons of the gene for LTC4 synthase 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 (Table I). In addition, the exons of LTC4 synthase and FLAP align identically with regard to the amino acids that they encode (Fig. 2). This fact also allows the deduced amino acids with the respective predicted secondary structures of the two molecules to be aligned as previously shown (25). The identical intron/exon organization of LTC4 synthase and FLAP suggests the evolution of these two molecules from the process of gene duplication, as has been proposed for the ancient gene family of glyceraldehyde-3-phosphate dehydrogenases, which share five identical intron positions (44). Identical intron/exon overlap has also been shown for the 5'-, 12-, and 15-lipoxygenase genes; the more closely related 12- and 15-lipoxygenases are on chromosome 17, and the less related 5-lipoxygenase is on chromosome 10 (37). Thus, although LTC4 synthase and FLAP are both 18-kDa integral membrane proteins involved in the synthesis of leukotrienes and appear by homology at both the protein and cDNA levels and by genomic organization to be related members within a novel family, their evolutionary divergence is significant in terms of intron size, 5'-flanking regions, and chromosomal location.

The human gene for LTC4 synthase has been localized with fluorescent in situ hybridization to the q35 region of chromosome 5 (Fig. 6). This finding contrasts with a report of the
localization of the human FLAP gene on chromosome 13 (45) and the 5-lipoxygenase gene on chromosome 10 (37). The long arm of the fifth chromosome has also been identified as the site at which many of the genes encoding growth factors, cytokines, and receptors relating to the asthmatic phenotype are localized. These include IL-3, IL-4, IL-5, and granulocyte-macrophage colony-stimulating factor, as well as IL-9, IL-13, and fibroblast growth factor-acidic, all localized within a gene cluster at 5q23–5q31 (46, 47). Receptors that have been localized more distally in the 5q31-q32 region include the β2 adrenergic receptor and the lymphocyte-specific corticosteroid receptor, whereas colony-stimulating factor receptor-1, monocyte colony-stimulating factor receptor, and platelet-derived growth factor receptor are in the 5q33-q35 region (47, 48). The most distal genes located in the 5q34-q35 region include dopamine receptor 1 and γ-butyric acid receptor (47).

The inflammatory changes of bronchial asthma demonstrated by biopsies are characterized by degranulation of mast cells as well as by infiltration of eosinophils and TH2 cells, all of which express markers of activation (49, 50). These findings implicate the products of genes residing on the long arm of chromosome 5. Interleukin-3, IL-5, and granulocyte-macrophage colony-stimulating factor not only regulate eosinophilopoiesis (28, 51, 52), but act on mature eosinophils to attenuate steroid-induced apoptosis (53). Furthermore, these cytokines convert eosinophils to a phenotype similar to that associated with disease in which the cells are primed for ligand-initiated generation of LTC4 and target cell cytotoxicity (54–56). IL-4 mediates immunoglobulin isotype switching in general and IgE biosynthesis by B cells in particular (57). IgE sensitizes mast cells and basophils through their high affinity receptors for allergen-specific activation, providing an additional mechanism for LTC4 generation. Importantly, IL-4 perpetuates the inflammatory reaction by favoring T cell maturation and differentiation to the TH2 phenotype, which provides IL-4 and the eosinophilopoietic cytokine triad (58).

In addition to the genomic localization of cytokines that amplify and perpetuate the asthmatic response to the long arm of the fifth chromosome, substantial clinical evidence supports the linkage of specific allelic oligonucleotide markers from this region of chromosome 5 to the atopic/asthmatic state. Atopy describes a heritable condition in which specific IgE is synthesized after exposure to specific allergens, and bronchial asthma is associated with bronchial hyperresponsiveness defined by compromised pulmonary function in response to environmental stimuli or concentrations of defined agonists that are inactive in the unaffected population. Bronchial hyperresponsiveness assessed by methacholine inhalation correlates with circulating levels of total IgE in individuals with asthma (59), and both of these co-inherited features demonstrate significant linkage to the IL-4 gene, localizing specifically to position 5q31.1 (60). Fibroblast growth factor-acidic and colony-stimulating factor 1 receptor have also been shown by linkage analysis to be disproportionately associated with bronchial hyperresponsiveness in sibling pairs (61). Specific polymorphisms have been identified in the enhancer sequences (a C to T exchange at position −590 from the open reading frame) of IL-4 and correlate with increased IL-4 level activity manifested by higher total serum IgE in atopic asthmatic kindreds (62). The immediate improvement of pulmonary function in individuals with asthma who receive an initial dose of agents that are devoid of intrinsic bronchodilatory activity but selectively attenuate the formation or action of the cysteinyl leukotrienes indicates that...
chronic overproduction of the cysteinyl leukotrienes occurs in the natural disease (4). The finding that the gene for LTC₄ synthase resides in the terminal region of the long arm of chromosome 5 adds another important candidate gene for asthma to the previously recognized cytokine genes clustered at the locus, i.e. one related to the generation of lipid mediators.

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