RESEARCH COMMUNICATION

The human leukotriene A₄ hydrolase gene is expressed in two alternatively spliced mRNA forms

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We identified a novel short (s-) mRNA of the human leukotriene-A₄ (LTA₄) hydrolase using sequential reverse transcriptase PCR mapping. The s-mRNA is generated by skipping an 83 bp exon located in the 3' coding region and suggests the expression of an LTA₄ hydrolase isoform with a calculated molecular mass of 59 kDa and a distinct C-terminus. Both LTA₄ hydrolase mRNAs

are constitutively expressed in blood cells, endothelial cells, smooth muscle cells, fibroblasts and tumour cells. The ratios of their mRNA expression levels are cell specific, with relatively high s-form mRNA expression in reticulocytes. Our data strongly suggest that the novel mRNA codes for the structurally related but distinct LTA₄ hydrolase isoenzyme that has been postulated.

INTRODUCTION

Leukotriene-A₄ (LTA₄) hydrolase (EC 3.3.2.6.) is a cytosolic enzyme that catalyses the conversion of LTA₄ into the potent pro-inflammatory eicosanoid LTB₄ [1,2]. Originally purified from leucocytes [3], LTA₄ hydrolase activity has been detected in a multitude of cells and tissues including erythrocytes [4]. Cloning of the human LTA₄ hydrolase cDNA from lung, placenta and spleen revealed a unique sequence [5,6]. The mature enzyme consists of 610 amino acids with a molecular mass of 69 kDa [7].

Protein sequence homology to zinc-containing metalloproteinases [8] and aminopeptidase activity [9,10] suggested a bifunctional role of the LTA₄ hydrolase enzyme, although an endogenous peptide substrate has not been identified. The aminopeptidase activity can be inhibited by metalloproteinase inhibitors [11,12] and is enhanced by chloride [13]. Disruption of the zinc-binding motif resulted in the virtual loss of both the LTA₄ hydrolytic activity and the aminopeptidase activity, and identified the amino acids corresponding to His-295, His-299 or Glu-318 as active residues [14].

Protein studies in lung tissue of guinea pigs [15] and in human airway epithelial cells [16], erythrocytes [17] and a B-lymphocytic cell line [18] suggested the presence of a structurally related, but distinct form of the LTA₄ hydrolase. Thus, an LTA₄ hydrolase isoenzyme was proposed that lacks enzyme inactivation by substrate [17] or heat [18], displays a slower time-course for product generation in combination with a distinct proteolytic cleavage pattern [19], and has an identical N-terminus with, and a highly similar overall amino acid composition to, the known LTA₄ hydrolase [18]. However, the putative isoenzyme has not yet been purified or conclusively characterized [19].

We report here the identification of an isoform of the human LTA₄ hydrolase mRNA by using a sequential reverse transcriptase (RT)-PCR mapping approach. The novel mRNA is generated by alternative splicing and suggests the expression of

an LTA₄ hydrolase isoform with a calculated molecular mass of 59 kDa and a distinct C-terminus.

EXPERIMENTAL

Cell preparations

Blood was obtained from healthy donors and specific cell populations were isolated as described previously [20–22]. Briefly, mononuclear cells (MNC), polymorphonuclear cells (PMNC) and red blood cells were purified by dextran sedimentation and subsequent centrifugation through Ficoll® [20–22]. The resulting cell fractions were platelet-free [21]. Monocytes (> 96 %) were obtained from freshly prepared MNC suspended in endotoxin-free RPMI-1640 medium/5 % fetal calf serum (Sigma, Munich, Germany) by adherence to polystyrene Petri dishes (Greiner, Nürtingen, Germany). After 15 min incubation (37 °C), non-adherent cells were removed by vigorous washing with pre-warmed PBS and adherent monocytes were Wright–Giemsa-stained for light-microscopy or lysed for RNA preparation (see below). T-lymphocytes were prepared by depletion of CD14⁺ cells from MNC using magnetic Dynabeads® (Dyna, Hamburg, Germany) coated with a monoclonal antibody (mAb) to CD14. Aliquots of 10⁷ MNC in PBS/2 % (w/v) fetal calf serum (Sigma) were incubated with an excess of mAb-CD14 beads (ratio 1 : 5) in a 400 µl volume under slow rotation at 4 °C. After 45 min the volume was increased and CD14⁺ cells were separated immunomagnetically. The remaining cells were depleted a second time for 20 min. mAb-CD14 double-depleted cells were free of beads and contained > 99 % lymphocytes. Pure platelets were obtained from platelet-rich plasma and freed of contaminating red cells by selective lysis [23,24]. Reticulocytes (CD71⁺ cells) were obtained from the red cell fraction, using magnetic Dynabeads® coated with an mAb to CD71 (Dyna). Immunomagnetically separated CD71⁺ cells were > 98 % pure as determined by light-microscopy of stained smears.

Abbreviations used: LT, leukotriene; M-MLV, Moloney murine leukaemia virus, RT, reverse transcriptase; HUVEC, human umbilical vein endothelial cells; VSMC vascular smooth muscle cells; BPS, branch-point sequence; s-mRNA, short mRNA; l-mRNA, long mRNA; mAb, monoclonal antibody; pl, isoelectric point; MNC, mononuclear cells; PMNC, polymorphonuclear cells.

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The sequences reported in this paper have been deposited in the GenBank data base under the accession numbers U43410 and U43411.

Human skin fibroblasts, rhabdomyosarcoma, osteosarcoma and U-937 cells were obtained from Dr. K. von der Helm (Department of Microbiology, Ludwig-Maximilians University, Munich, Germany). Human umbilical vein endothelial cells (HUVEC) and vascular smooth muscle cells (vSMC) were gifts from Dr. A. J. Marcus (Hematology-Oncology, Cornell University, New York, NY, U.S.A.), and Dr. R. Ross (Department of Pathology, University of Washington, Seattle, WA, U.S.A.) respectively.

Extraction of nucleic acids and cDNA synthesis

Total RNA was isolated by lysing aliquots of 5×10^5 cells in RNA extraction buffer containing 0.5% (w/v) Triton X-100, 2% (w/v) SDS, and 1000 units/ml placental RNase inhibitor (Boehringer Mannheim, Germany), followed by proteinase K digestion (200 mg/ml, Boehringer) and phenol/chloroform extraction [20–22]. Aliquots of 500 ng total RNA were reverse-transcribed into cDNA using Moloney murine leukaemia virus RT (Gibco-BRL, Nürtingen, Germany) and dT₁₅ priming according to the manufacturer's protocol. Highly pure genomic DNA with fragments larger than 50 kb was prepared from 10⁸ PMNC using the Qiagen DNA-Prep kit (Qiagen, Hilden, Germany). Yields and purity of RNA and DNA were determined spectrophotometrically and by agarose-gel electrophoresis respectively.

Oligonucleotides

20-mer oligonucleotide primers (Mikrogen, Munich, Germany) were designed according to the published LTA₄ hydrolase cDNA [5,6]. Primers were selected for RT-PCR to allow sequential mapping of the complete cDNA in overlapping subsegments of 120 bp–1.8 kb in size (Figure 1). Two nested primer sets (see Figure 1: external set, 1479 and 1767; internal set, 1500 and 1746) allowed for competitive RT-PCR amplification and were used for the relative quantification of steady-state mRNA

expression levels. Additional primers, used for amplification of the intron regions, were: 5' intron: forward (5'-TCTCATC-AATTGAATGAGTT-3') and reverse (5'-CAGAATTGTTA-ATGGCATTG-3'); 3' intron: forward (5'-CAACTTCAATGCCATTAACA-3') and reverse (5'-CCACTTGGATTGAATGCAGA-3'). In some experiments isoform-specific reverse primers were used that allowed the selective amplification of the short (s) and long (l) mRNAs: s-form (5'-AGAGCCGCAGCCATCCTCTG-3'); l-form (5'-TTTCAGAATTGTTAATGGCATTG-3')

Genomic PCR

Amplification of introns was performed with 50, 100 and 200 ng of human genomic DNA respectively, in a reaction volume of 100 μ l containing 25 nM oligonucleotide primers, 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 2.5 mM MgCl₂, 25 mM dNTP, 5 units of Amplitaq[®] DNA polymerase (Perkin-Elmer Cetus, Überlingen, Germany) and 5 units of Taq Extender[®] PCR additive (Stratagene, Heidelberg, Germany). All reaction mixes were covered with mineral oil and cycled 30 times at 94 °C (20 s), 55 °C (30 s), 72 °C (1–15 min) after an initial denaturation step (94 °C, 1 min). This method, in our hands, allowed for the amplification of genomic DNA fragments > 23 kb (results not shown).

RT-PCR and quantification of mRNA levels

RT-PCR amplifications were performed in a 50 μ l total volume containing 10 mM Tris/HCl (pH 9.0), 50 mM KCl, 2.0 mM MgCl₂, 10 mM dNTP, 1.25 units of Amplitaq DNA polymerase (Perkin-Elmer) and 40 nM–1.5 μ M primers. A typical PCR cycle profile was: 94 °C (25 s), 55 °C (30 s), 72 °C (30 s). The RT-PCR technique for quantification of relative mRNA levels has been described previously [20–22]. Optimal conditions for nested sets of LTA₄ hydrolase primers were established to confirm log-linear amplification. Steady-state mRNA levels were normalized to the expression of β -actin. Amplification products were separated on

Figure 1 (A) RT-PCR mapping of the LTA₄ hydrolase mRNA and (B) structure of the two LTA₄ hydrolase mRNAs and their derived C-terminal amino acid sequences

(A) Locations of forward () and reverse () primers used for complete RT-PCR mapping of the LTA₄ hydrolase cDNA. Numbers indicate primer locations (5'-end) and refer to the numbering of the cDNA published by Minami et al. [6]. (B) The short LTA₄ hydrolase mRNA variant (s-mRNA) differs from the long form (l-mRNA) in the absence of an alternative 83 bp exon located in the 3'-region of the coding sequence (filled box). Skipping of the alternative 83 bp exon leads to a frameshift that introduces a preterminal stop (TGA) into the LTA₄ hydrolase s-mRNA coding sequence. The derived amino acid sequence of the s-form mRNA variant predicts a truncated LTA₄ hydrolase isoform in which the 103 C-terminal amino acids of the canonical LTA₄ hydrolase are replaced by 22 novel amino acids (shaded box).

Figure 2 Expression of the LTA₄ hydrolase short (s-) and long (l-) mRNA variant in MNC.

Human MNC were obtained *ex vivo* from three different individuals (I–III) at 0, 1 and 6 weeks. Total RNA was extracted and quantitative RT-PCR analysis (see Experimental section) was performed that demonstrated constitutive expression of both LTA₄ hydrolase mRNA variants. Ratios of expression levels of the l-mRNA (246 bp) and the s-mRNA (163 bp) forms ranged between 5.9 and 14. The primers used allowed for the simultaneous (competitive) amplification of both LTA₄ hydrolase mRNA variants. M, DNA markers

ethidium bromide-stained agarose gels (1.5 %) and photographed under UV light (Polaroid 665 film). The negatives were analysed densitometrically (UltraScan laser densitometer; Pharmacia LKB, Germany). Relative levels of LTA₄ hydrolase s-mRNA were related to the respective values of l-mRNA. All quantitative PCR analyses were performed with identical lots of reagents and stock solutions; data represent means of at least three separate measurements.

DNA sequencing

PCR products (Figures 1 and 2) were resin-purified, ligated into pGEM-T vectors and cloned into JM109 host cells (Promega, WI, U.S.A.; Serva, Germany). Plasmid DNA was prepared using the Qiaprep-spin plasmid kit (Qiagen). Both strands of cloned PCR segments were sequenced using the Sequenase 2.0 kit (USB, Cleveland, OH, U.S.A.), SP6 and T7 universal primers (Promega) or target-sequence-specific oligonucleotides (see Figure 1 above). For computer-assisted sequence analyses and homology searches [25] MacDNAsis Software Version 2.0 was applied.

RESULTS AND DISCUSSION

Sequential RT-PCR mapping of LTA₄ hydrolase transcripts, using primers specific for the published cDNA [5,6], revealed the expression of two distinct LTA₄ hydrolase mRNA forms in

human MNC (Figures 1 and 2). Detailed PCR analysis using multiple primer combinations (Figure 1A) suggested heterogeneity in the 3'-mRNA region that resulted in the tandem amplification of two fragments. The larger amplification products had predicted fragment sizes (ranging between 0.2 and 1.9 kb). The co-amplified products were approx. 80 bp smaller in size (Figures 1 and 2). A selected number of the RT-PCR amplification products were cloned and sequenced. These included 1863 bp, 1274 bp and 842 bp segments (obtained by primers 62, 651, 1083, and 1925 respectively, Figure 1A) and their respective co-amplification products.

Sequence analysis of the fragments with predicted sizes showed complete identity with the coding sequence of the human LTA_4 hydrolase cDNA obtained from lung or spleen tissue [5,6]. In contrast, the sequences of the co-amplified products were identical with this sequence, with the exception of an 83 bp segment (positions 1598–1681) that was absent in the 3' coding region (Figure 1B). Deletion of the 83 bp segment leads to a +2 frameshift, resulting in a pre-terminal stop after 22 amino acids (Figure 1B). Thus human MNC express a novel short LTA_4 hydrolase mRNA variant (s-form) that suggests the expression of an LTA_4 hydrolase isoform with an alternative C-terminus (Figures 1 and 2).

With the exception of the 83 bp segment, the novel LTA₄ hydrolase s-mRNA is identical with the LTA₄ hydrolase mRNA previously described [5,6]. This strongly suggests that the s-form mRNA is the result of alternative exon splicing rather than transcription from a different gene. To test whether the LTA₄ hydrolase gene is a single-copy gene and to characterize the putative flanking introns, we applied a genomic PCR approach using primers that were localized in the adjacent exons and in the alternatively expressed 83 bp segment respectively. Upstream intron amplification resulted in a single 2.6 kb segment (Figure 3), and amplification of the flanking downstream intron yielded a single band of 1.0 kb in size (Figure 3), indicating the presence of only one LTA₄ hydrolase gene. Both products were cloned and sequenced. Sequence analysis verified the predicted exon/intron borders (Figure 3). The intron sizes and exon/intron architecture demonstrated here were confirmed by an independent report published during the preparation of this manuscript [26].

Sequence analysis of the identified exon/intron borders and the intron flanking regions revealed consensus sequences for *cis*-acting elements (Figure 3) that are required for the processing of pre-mRNA [27]. The initial and terminal dinucleotides of either of the flanking LTA, hydrolase introns conform to the GT-AG

5' - **CTCCAGAGG** [**GTAAGTACTAGCTCAAAATCTTAGAAATTA**AAATGGACAGAAAGGCACAGCATTTATGTCTCACCA
ACTTGGCAAAAGATTATTAATGTGGAACATAATGGGGAGAAATCATTTATAAAATTTATTCATTCTCTTAAAAAAGCAT
TGAATGCTTACTGTGTTTATATTTGTCTAAATATGAAATATGTAGAAGAGGATAT / - 2.0 kb - / AAAGGTAAAAATAT
CCCTGTGCTCCGAGTAGTAATACCTCAAGCGAATAAGTTGCACAGAATTTACTTTAGTGTGCAATTTGTTCTTTTAT
TTTTATCTGACTTATTTGTATATTATAAAATATTTAGGATTTTAAATCTGGCTATGCATTTCTCTCCAGTCTCTTCTTCC
AAGGAATCCCTGGAGATACGGTATACTTCTTTATTTTGCTCTTAG] [**GCACCTCTTCCATTGGGGCACATAAGCGGA**
ATGCAAGAGGGTACAACCTCAATGCCATTAAACAATCTTGAAATACGATTTCAG] [**GTACATCATTTTAACTTGCTCTGTG**
GAAGAAAGCAAAAACATAAACCTCTTTGAAAGATGGGAAAGGGAATGCAGAGTATGAGACATCTTGAGTCTTGAGAAAT
TCCTTGTCAAACTGACTGCTCTATATAAATACTATAGTGGTATTTAAGGAATAGTTGCAATATGTAATCTCGTGATGAT
TAATCTTTTGTGTAGACTTTGCT / - 0.5 kb - / TGATCTTTTCAAGAACCGCTCTCTCTTCTTCTTCCACCAATTAT
TAAATGTATATATAGGATTTGAGAGTCTCAGAATCAAGACATACCATAAAGCGGTGTACAGTATAACTATAGTGGGT
TATTCATATCTACTACAGAAGTAAATTAAGATATACCTGTATATCTGCCCTCTGCTGATTTGCTGACATTCATCTCCATGCTCCC
CCCAATCTCTCTTTTAG] [**ATGGCTCGG-3'**

Figure 3 Nucleotide sequence of the alternatively spliced LTA, hydrolase exon and its flanking intron regions

The alternative 83 bp exon (bold) is flanked by a 2.5 kb intron (upstream) and a 1 kb intron (downstream). Intron sequences are shown in roman; [] indicate intron/exon boundaries. Splice donor and acceptor sites are underlined. Putative BPSs (YNYTRAY) are outlined. The nucleotide sequences of the adjacent upstream and downstream exons respectively, are partially shown (*italic*).

Figure 4 Tandem expression of the LTA_4 hydrolase s- and l-mRNAs in various human cells

mRNA expression levels of each cell type were analysed by quantitative RT-PCR (see Experimental section) using l- and s-form-specific primers. The ratios of l-form:s-form mRNA levels are shown.

rule [28]. The 5' splice donor site of both introns is homologous with the defined consensus AG/GTRAGT, and the conserved 3'-splice acceptor sites (NYAG/R) are preceded by a pyrimidine (Y)-rich tract (Figure 3). Thus, the DNA domains that are typically required for identifying the exon/intron borders [27] are well conserved. Furthermore, putative branch-point sequences (BPS) [29], which are crucial for completing the constitutive removal of introns, are present in both flanking introns and are positioned typically within 18–40 nucleotides upstream of the respective acceptor sites (Figure 3). The putative BPS (YNYTRAY) present in the upstream flanking intron is well conserved. In contrast, both putative BPSs found in the downstream intron (Figure 3) contain mismatches that might affect the splicing mechanism and the frequency of exon splicing [27].

Time-course analyses of LTA_4 hydrolase steady-state mRNA levels over a 6-week time period showed that both LTA_4 hydrolase mRNA isoforms are constitutively expressed in human MNC (Figure 2). Quantitative RT-PCR analysis of the mRNA expression levels in MNC, obtained from three individuals, demonstrated that the smaller isoform is expressed at lower levels compared with the l-form (between 10 and 20%) (Figure 2). Relative quantification of mRNA levels was performed using a nested RT-PCR approach (see the Experimental section).

RT-PCR analysis of a selected number of cells and cell lines showed tandem expression of the two LTA_4 hydrolase mRNA isoforms in all cells investigated, including monocytes, peripheral lymphocytes ($CD14^+$ cells), neutrophils, platelets and reticulocytes ($CD71^+$) (Figures 2 and 4). Both mRNA variants were also expressed in HUVEC, vSMC and skin fibroblasts (Figure 4). Furthermore, several cultured tumour cell lines, derived from solid tumours (rhabdomyosarcoma, osteosarcoma) and leukaemia cells (U937), expressed both LTA_4 hydrolase transcripts (results not shown). Thus, our results make the ubiquitous expression of the two LTA_4 hydrolase mRNA forms likely.

Quantification of steady-state mRNA levels in white blood cell populations, platelets and SMC showed a generally higher expression of the l-form compared with the s-form (Figures 2 and 4). In contrast, reticulocytes, fibroblasts and HUVEC expressed almost equal levels of both transcript isoforms (Figure 4). Thus, the ratios of the s- and l-form LTA_4 hydrolase mRNA steady-state levels varied considerably depending on the cell type (Figures 2 and 4), suggesting that the mRNA expression and/or

stability of both mRNA isoforms are regulated in a tissue-specific manner.

LTA_4 hydrolase mRNA appears as a single hybridization signal of ~2.3 kb in conventional Northern blot analysis (results not shown; see [5,6]). However, considering the low resolution of this method, the minimal size difference between the two LTA_4 hydrolase mRNAs and the lower expression levels of the s-form observed in most cells (Figures 2 and 4), it is conceivable that the single band detected by Northern blot analysis represents the two LTA_4 hydrolase mRNA isoforms.

The expression of the short LTA_4 hydrolase mRNA we identified (Figures 2 and 4) suggests the expression of an LTA_4 hydrolase isoenzyme that is structurally highly homologous with the known LTA_4 hydrolase (Figure 1). This is consistent with several independent reports that observed the activity of a functionally and structurally related, but catalytically distinct, LTA_4 hydrolase [15–19]. The inactivation patterns of LTA_4 hydrolase enzyme preparations, obtained from human erythrocytes, indicated the presence of a major (85%) and a minor (15%) LTA_4 hydrolase activity respectively [17]. In contrast with the enzyme fraction that contained the major activity, the fraction with the minor activity was resistant to substrate inactivation [17]. Moreover, LTA_4 hydrolase purified from a transformed B-cell line displayed a biphasic heat-inactivation pattern that strongly suggested the presence of two catalytically divergent isoenzymes [18]. Determination of the N-terminal sequence and the amino acid composition revealed no significant differences between the putative isoenzymes [18]. Therefore, it has been proposed that the two LTA_4 hydrolase isoenzymes might exhibit a homologous secondary structure with a limited difference in their C-terminal regions [15]. The novel LTA_4 hydrolase s-form mRNA we report here predicts a polypeptide of 533 amino acids with a different C-terminus and a calculated molecular mass of 59.598 kDa (Figure 1B). Expression of an LTA_4 hydrolase isoenzyme with a lower molecular mass would be in accordance with the molecular mass range that has been reported for the LTA_4 hydrolase purified from human erythrocytes [4]. The calculated isoelectric point (pI) of the LTA_4 hydrolase s-form is 5.24 (l-form: 5.76). Interestingly, two catalytically active forms of LTA_4 hydrolase that displayed different pIs of 5.4 and 5.7 respectively, have been observed in the guinea pig lung [15].

The sequence that codes for the zinc-ion-binding motif and the peptidase active site is identical in the s-form and l-form LTA_4 hydrolase mRNAs (Figure 1). Therefore, the LTA_4 hydrolase and aminopeptidase activities might very well be intact. However, the new C-terminus might modify and/or add new biochemical characteristics. Expression studies as well as the generation of specific antibodies that will allow further characterization of the novel LTA_4 hydrolase mRNA isoform are under way.

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