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Biochemistry

Molecular cloning, primary structure, and expression of the human platelet/erythroleukemia cell 12-lipoxygenase

(arachidonate metabolism/isoenzyme/phorbol 12-myristate 13-acetate)

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ABSTRACT **The major pathway of arachidonic acid metabolism in human platelets proceeds via a 12-lipoxygenase enzyme; however, the biological role of the product of this reaction, 12-hydro(pero)xyeicosatetraenoic acid [12-H(P)ETE], is unknown. Using a combination of the polymerase chain reaction and conventional screening procedures, we have isolated cDNA clones encoding the human platelet/human erythroleukemia (HEL) cell 12-lipoxygenase. From the deduced primary structure, human platelet/HEL 12-lipoxygenase would encode a M_r 75,000 protein consisting of 663 amino acids. The cDNA encoding the full-length protein (pCDNA-12lx) under the control of the cytomegalovirus promoter was expressed in simian COS-M6 cells. Intact cells and lysed-cell supernatants were able to synthesize 12-H(P)ETE from arachidonic acid, whereas no 12-H(P)ETE synthesis was detected in mock-transfected cells. A single 2.4-kilobase mRNA was detected in erythroleukemia cells but not in several other tissues and cell lines evaluated by Northern blot analysis. Comparison of the human platelet/HEL 12-lipoxygenase sequence with that of porcine leukocyte 12-lipoxygenase and human reticulocyte 15-lipoxygenase revealed 65% amino acid identity to both enzymes. By contrast, the leukocyte 12-lipoxygenase is 86% identical to human reticulocyte 15-lipoxygenase. Sequence data and previously demonstrated immunochemical and biochemical evidence support the existence of distinct 12-lipoxygenase isoforms. The availability of cDNA probes for human platelet/HEL cell 12-lipoxygenase should facilitate elucidation of the biological role of this pathway.**

In 1974, Hamberg and Samuelsson (1) described the characterization of a platelet 12-lipoxygenase enzyme (EC 1.13.-11.31) that was able to insert molecular oxygen stereospecifically into position C-12 of arachidonic acid to yield (12*S*)-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid (12-HPETE) (1). Platelets also transform arachidonic acid to the potent vasoconstrictor and aggregatory eicosanoid thromboxane A_2. Although the biological significance of this latter pathway has been clearly established (2, 3), the role of the platelet 12-lipoxygenase continues to be a perplexing issue. The use of nonspecific lipoxygenase inhibitors and experiments employing the 12-HPETE metabolite 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) have failed to yield definitive answers as to the physiological role of the 12-lipoxygenase (4).

After its original discovery in platelets, 12-lipoxygenase activity was found in several other tissues, including porcine leukocytes, human and mouse epidermal cells, and bovine tracheal epithelial cells (5–7). The enzyme subsequently was purified to near homogeneity from the cytosolic fractions of porcine leukocytes (8) and from bovine tracheal epitheal cells (7), yielding proteins of M_r 72,000. Monoclonal antibodies to both human platelet and porcine leukocyte 12-lipoxygenase have been prepared (9). Differences in immunochemical (antibody crossreactivity) and biochemical (substrate specificity, reaction kinetics, and heat stability) parameters have indicated that the bovine platelet and leukocyte 12-lipoxygenases are likely to be distinct isoforms (9).

The amino acid sequences for several other lipoxygenases have been deduced recently from the cloning of their respective cDNAs (10–12). Although platelets contain minute amounts of vestigial RNA, the polymerase chain reaction (PCR) has been employed successfully to amplify platelet-specific mRNAs (13). Using a combination of this approach and traditional screening of cDNA libraries derived from a human erythroleukemia (HEL) cell line that displays platelet-like characteristics (14), we have successfully isolated cDNA clones encoding a 12-lipoxygenase, deduced its primary structure, and confirmed its identity by expression in eukaryotic cells.*

MATERIALS AND METHODS

λgt11 cDNA libraries from phorbol 12-myristate 13-acetate (PMA)-treated HEL cells (15) were generous gifts from E. Lapetina (Wellcome; library 1) and G. J. Roth (Division of Hematology, Veterans Administration Hospital, University of Washington, Seattle; library 2). Restriction endonucleases, other nucleic acid-modifying enzymes, M13 vectors, and sequencing reagents were obtained from Promega, Boehringer Mannheim, and Pharmacia. Multiprime DNA-labeling and GeneAmp kits were from Amersham and Perkin–Elmer/Cetus, respectively. Radioactive nucleotides were obtained from New England Nuclear. Oligodeoxynucleotides were synthesized by Midland Certified Reagent (Midland, TX) and were either purified by OPC reverse-phase cartridges (Applied Biosystems) or used directly.

PCR. Total RNA was prepared (16) from washed human platelets obtained from ≈10 ml of blood. An aliquot (1/10th) was taken for first-strand cDNA synthesis (final volume, 25 μl) using avian myeloblastosis virus reverse transcriptase with the downstream oligonucleotide (25–50 pmol) as primer. The mixture was diluted to 100 μl and adjusted to PCR conditions [10 mM Tris·HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.2 mM each dNTP, 25–50 pmol of each primer set, and 2.5 units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase)], and amplification of target DNA was performed as follows: denaturation at 94°C for 1 min; annealing at 37°C for 1 min 45 sec; extension at 72°C for 3 min for the first three cycles, followed either by 30 cycles with a 50°C annealing temperature, for clone PL12lx or by 30 cycles of

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Abbreviations: HEL, human erythroleukemia; HPETE, hydroperoxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35418).

94°C, 45 sec; 45°C, 45 sec; 72°C, 1 min for clone PL12lxA. The amplified products were electrophoresed in 1.2% agarose gels and the expected target DNA was excised and purified by glass-powder elution (Gene Clean kit, BIO 101). The DNA (1/100th) was subjected to a second round of amplification as described above.

Hybridization Screening. The 0.26-kilobase (kb) PCR-amplified DNA, PL12lx, was ^{32}P-labeled by the random primer method (17) and used to screen λgt11 HEL cell cDNA libraries by methods previously described (18).

Subcloning and Sequencing. The *Eco*RI inserts of positive phage clones were purified and sized by electrophoresis in 1.2% agarose gels. Inserts and restriction fragments were subcloned into M13mp18 and M13mp19 vectors and sequenced by the dideoxy chain-termination method (19) using the M13 universal primer or primers derived from sequenced regions.

Northern Blot Analysis. RNA was size-fractionated by electrophoresis in 1% agarose gels containing 0.22 M formaldehyde, transferred to nitrocellulose, and hybridized with the ^{32}P-labeled 0.26-kb PL12lx DNA by methods previously described (18).

Expression of Cloned Human 12-Lipoxygenase DNA. A DNA expression vector encoding 12-lipoxygenase was constructed as follows. (*i*) The *Eco*RI insert of phage clone HEL12lxG was modified at its 3′ end to delete the 3′ noncoding region and incorporate a *Sal* I site by PCR to create a 1.15-kb *Eco*RI–*Sal* I fragment. This DNA was subsequently cloned into the *Eco*RI and *Xho* I sites of the expression vector pCDNA1 (Invitrogen, San Diego) to yield pCDNA-3′12lx. (*ii*) The 0.9-kb *Eco*RI insert of clone HEL12lxH was cloned into *Eco*RI-cleaved, calf alkaline phosphatase-treated pCDNA-3′12lx to give pCDNA-12lx. The orientation of the *Eco*RI fragment was checked by *Sac* I digestion. pCDNA-12lx (10–20 μg) was introduced into COS-M6 cells (seeded at 10^6 cells per 100-mm plate) by a standard calcium phosphate transfection procedure (20). Cells were harvested 60 hr after transfection and aliquots were assayed for 12-lipoxygenase activity. Briefly, intact cells or the 10,000 × *g* supernatants from sonicated cells in 50 mM Tris·HCl (pH 7.4) or phosphate-buffered saline containing 1 mM EDTA were incubated with 100 μM arachidonic acid (10 nmol) and a lipoxygenase activator, 13-hydroperoxyoctadecadienoic acid (0.5 nmol) for 10 min at 37°C in a final volume of 100 μl. The reactions were stopped and HPLC analysis was performed as described (21).

Sequence Analysis. DNA sequence analysis was performed with programs developed by the University of Wisconsin Genetics Computer Group (22).

RESULTS

Isolation of a 12-Lipoxygenase Clone by PCR Cloning. When we began this study, no sequence information about the 12-lipoxygenase(s) was available. However, it was known that 5-lipoxygenase and 15-lipoxygenase share certain highly homologous regions (11). Based on the hypothesis that 12-lipoxygenase contains similar homologous segments, a PCR cloning approach was instituted to isolate a clone encoding platelet 12-lipoxygenase. Oligonucleotide primers corresponding to these homologous segments were synthesized (see legend to Fig. 2) and used in a PCR with platelet cDNA as a template. A 0.26-kb amplified DNA (PL12lx; Fig. 1) was purified and sequenced and was found to contain a single open reading frame. The translated sequence revealed a sequence with 76% and 60% amino acid identity with human 15-lipoxygenase and human 5-lipoxygenase, respectively.

Isolation and Characterization of Additional 12-Lipoxygenase Clones. Isolation of platelet cDNA clones corresponding to regions 5′ and 3′ to PL12lx by means of a single-sided PCR approach was unsuccessful. Since platelet cDNA libraries are extremely difficult to prepare, we screened cDNA libraries derived from a cell line displaying platelet-like characteristics, namely, PMA-treated human erythroleukemia cells (14). These cells were demonstrated to contain 12-lipoxygenase activity (data not shown). One clone (HEL12lx3) was obtained after screening ≈3 × 10^5 clones of library 1, and 2 clones (HEL12lxG and -H) were isolated after screening ≈3 × 10^5 phage clones of library 2. The 1.5-kb cDNA insert of HEL12lx3 contained the complete 3′ end of the 12-lipoxygenase cDNA, including the coding region for the carboxyl-terminal 380 amino acids and 314 base pairs (bp) of 3′ noncoding DNA (Figs. 1 and 2). A polyadenylylation signal (AATAAA) was located 15 bp upstream of a 35-bp poly(A) tail.

The *Eco*RI insert of phage clone HEL12lxG was nearly identical with the insert of HEL12lx3, extending 23 bp upstream and ending 2 bp after the consensus polyadenylylation signal (Figs. 1 and 2). Phage clone HEL12lxH contained two *Eco*RI inserts of 0.9 kb and 0.3 kb. When sequenced and translated, both inserts were found to contain sequences homologous to other lipoxygenases and could be readily aligned to give an open reading frame of 854 bp, encoding the amino-terminal 284 amino acids and having 41 bp of 5′ noncoding region. The combined sequence information of the *Eco*RI phage clone inserts indicates that the 12-lipoxygenase cDNA would encode a protein of 663 amino acids with M_r 75,000.

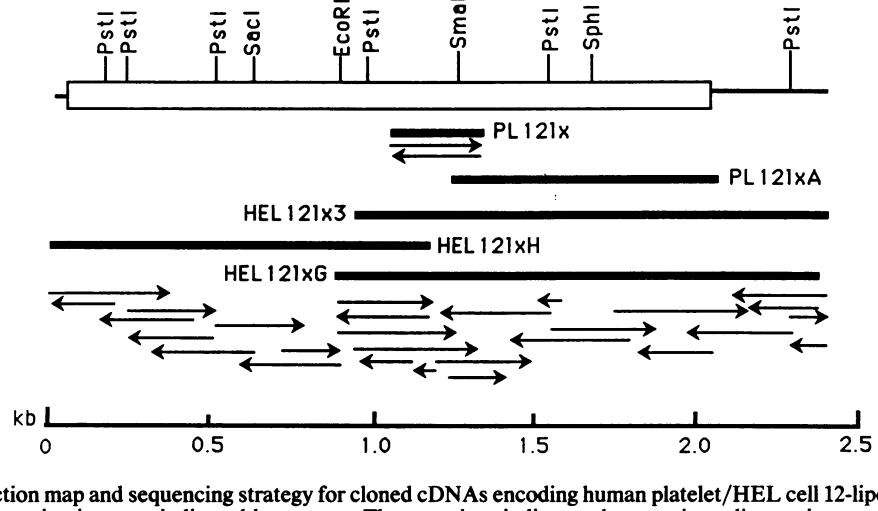

FIG. 1. Partial restriction map and sequencing strategy for cloned cDNAs encoding human platelet/HEL cell 12-lipoxygenase. Direction and extent of sequencing determinations are indicated by arrows. The open box indicates the protein-coding region.

FIG. 2. Nucleotide sequence of 12-lipoxygenase cDNAs and the predicted amino acid sequence. Nucleotides and amino acids are numbered beginning with the ATG initiator codon. Nucleotides to the 5′ side are designated by negative numbers. The consensus polyadenylylation signal is underlined. The dashed underlined regions represent the two regions chosen for oligonucleotide primers used in the PCR cloning of the platelet PL12lx cDNA clone. The upstream primer was 5′-TAGTCGACTGGCTTC/TTGGCCAAAIICTGGGTGCG-3′ and the downstream primer was 5′-CCAAGCTTCTGCACGTGNCCNCCICCIGT-3′, where I is deoxyinosine and the underlined regions represent restriction sites added to facilitate subcloning. The base at position 889 of clones HEL12lx3 and HEL12lxG was guanine, but was adenine in clone HEL12lxH. This may represent an allelic variant resulting in the change Ala-297 → Thr-297 (boxed). Alternatively, it may represent a cloning artifact.

After the complete cDNA sequence of the phage clone inserts obtained from the λgt11 HEL cDNA libraries was determined, two oligonucleotides were synthesized and used in a PCR to amplify a region of platelet 12-lipoxygenase cDNA corresponding to the carboxyl-terminal 267 amino acids (clone PL12lxA; Figs. 1 and 2). Combined sequence information from platelet clones PL12lxA and PL12lx indicates that these clones are identical to the HEL cell-derived clones, and they are presumed to encode an identical 12-lipoxygenase.

Expression of 12-Lipoxygenase. The plasmid vector pCDNA-12lx, carrying the 12-lipoxygenase cDNA under the control of the cytomegalovirus promoter, was used to express the cDNA in COS-M6 cells. Broken cell preparations obtained 60 hr after transfection were found to synthesize large amounts of products that comigrated with authentic standard 12-HETE and 12-HPETE on reversed-phase HPLC (Fig. 3) and normal-phase HPLC (data not shown). The products also displayed UV spectra identical to those of the standard compounds (data not shown). No lipoxygenase products were detected in preparations of normal COS-M6 cells or mock-transfected cells. The product with the same retention time as 12-HPETE, when treated with triphenylphosphine, was converted to a compound with the same chromatographic properties as 12-HETE, indicating reduction of the hydroperoxide moiety. These results are consistent with the activity of a 12-lipoxygenase enzyme. When arachidonic acid was added to intact cells, 12-HETE was formed but not 12-HPETE. Presumably, the 12-HPETE was completely reduced by cellular peroxidases.

Northern Blot Analysis. Poly(A)$^+$ RNA from normal and PMA-stimulated HEL cells, human placenta, and human liver and total RNA from human lung tissue were subjected to blot analysis using the ^{32}P-labeled PL12lx clone (Fig. 4). Hybridization to a discrete 2.4-kb mRNA was observed primarily in the lane containing PMA-stimulated HEL cell RNA. After prolonged autoradiographic exposure (4 days) a band could also be visualized in the lane containing normal HEL cell RNA. However, no hybridization was observed with the other samples. In addition, when RNA from several other tissues and cell lines, including human adrenal gland, UCLA-P3 human lung epithelial cells, rat and hamster pancreatic cells,

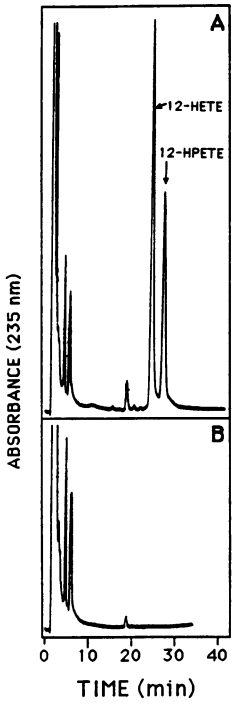

FIG. 3. Reversed-phase HPLC of lipoxygenase products from pCDNA-12lx-transfected (*A*) or mock-transfected (*B*) COS-M6 cells. Aliquots of 10,000 × *g* supernatants from sonicated cells were assayed for lipoxygenase activity. pCDNA-12lx-transfected cells (3.2×10^6) synthesized ≈2.5 nmol of 12 H(P)ETE, whereas the mock-treated cells (3.2×10^6) did not produce lipoxygenase products. The retention times of various lipoxygenase products were as follows: 12-HETE, 24.2 min; 12-HPETE, 27.6 min; 15-HETE, 20.2 min; and 5-HETE, 28.9 min. The small peak at 18.8 min is 13-hydroxyoctadecadienoic acid, a metabolite of the lipoxygenase activator, 13-hydroperoxyoctadecadienoic acid (retention time, 21 min).

and human aorta, was examined for the presence of 12-lipoxygenase mRNA, no hybridization was detected (data not shown).

DISCUSSION

Human platelets synthesize large amounts of arachidonic acid-derived 12-H(P)ETE. However, its biological function is poorly understood. 12-HETE was reported to enhance the proliferation of rat aortic smooth muscle cells (23) and the mitogenic potential of epidermal growth factor in NIH 3T3 mouse fibroblasts (24). 12-HETE is capable of inducing endothelial cell retraction (25) and enhances the surface expression of a protein immunologically related to the platelet glycoprotein gpIIb/IIIa complex on tumor cells (26). We are interested in exploring the role of the platelet 12-lipoxygenase in modulating platelet/vessel wall interactions. Therefore, in the present study, we have taken steps to obtain the molecular tools necessary for this purpose.

The cDNA for human platelet/HEL cell 12-lipoxygenase has been isolated and its identity verified by expression of an active 12-lipoxygenase in COS-M6 cells. Platelet/HEL 12-lipoxygenase displays a distinct relationship with other mammalian lipoxygenases (Fig. 5). It is 65% identical to both porcine leukocyte 12-lipoxygenase (78% similarity, including conservative substitutions) and human reticulocyte 15-lipoxygenase (80% similarity) and 40% identical to human 5-lipoxygenase (62% similarity). A lesser relationship (23–25% identity, 45% similarity) to the soybean and pea seed lipoxygenases is observed. In contrast, porcine leukocyte 12-lipoxygenase is 86% identical (92.5% similar) to human reticulocyte 15-lipoxygenase, indicating that these two enzymes are much more closely related. Platelet/HEL 12-lipoxygenase, therefore, is likely to represent a distinct isoform from the porcine leukocyte enzyme (12), the counterpart of which does not appear to exist in human leukocytes. Thus, it has recently been postulated (27) that human leukocyte 12-HETE production may arise from an intrinsic ability of eosinophil 15-lipoxygenase to generate this compound. Platelet/HEL 12-lipoxygenase exhibits an overall hydrophobicity profile that is similar to those of other members of the lipoxygenase family, and it shares many conserved basic and acidic residues (Fig. 5). These residues are likely to be fundamental to the structure–function properties of these enzymes, including the putative iron-binding domain (21). There are no strong predictions for membrane-spanning domains; this is consistent with the cytosolic localization of the enzyme (9).

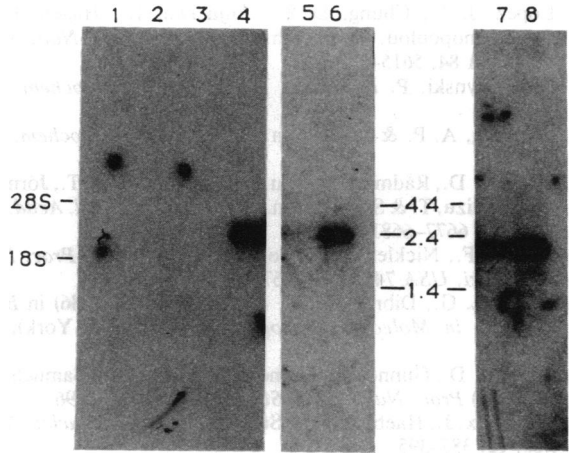

FIG. 4. Autoradiograms of RNA blot analysis. Human poly(A)$^+$ RNA (total RNA for the lung sample) from various tissues and cells was electrophoresed in 1% agarose gels containing 0.22 M formaldehyde, blotted onto nitrocellulose, and hybridized with the ^{32}P-labeled PL12lx clone (lanes 1–6) or with a ^{32}P-labeled rat β-actin probe (lanes 7 and 8). Lanes: 1, placenta (2 μg); 2, lung (10 μg); 3, liver (2 μg); 4, PMA-stumulated HEL cell (2 μg); 5 and 7, unstimulated HEL cell (0.5 μg); 6 and 8, PMA-stimulated HEL cell (0.5 μg). The samples in lanes 5 and 6 were electrophoresed in a different gel than those of lanes 1–4. The blot corresponding to samples 7 and 8 represents a reprobing of the same membrane as samples in lanes 5 and 6. Positions of 28S and 18S ribosomal RNAs are indicated at left. RNA standards (BRL) were electrophoresed in adjacent lanes and are indicated in kilobases.

12-Lipoxygenase protein has been identified in many different porcine tissues by using an enzyme-linked immunoassay with monoclonal antibodies raised against the porcine leukocyte enzyme (28). These antibodies did not recognize the bovine platelet 12-lipoxygenase but did immunoprecipitate the bovine leukocyte enzyme (9). In contrast, the bovine platelet 12-lipoxygenase crossreacted only with antibody against the human platelet enzyme. We were able to detect 12-lipoxygenase mRNA only in HEL cells and not in any other tissues or cell lines that we examined by Northern blot analysis. However, two clones (PL12lx and PL12lxA) were obtained by PCR analysis, providing evidence for the existence of the 12-lipoxygenase transcript in human platelets. Control experiments indicated that these clones were platelet RNA-derived and were not due to genomic DNA contamination (data not shown). Perhaps the platelet/HEL 12-lipoxygenase is a unique isoform, expressed only in these cells, and the 12-lipoxygenase activity and protein reported to be present in other tissues are due to expression of an alternative isoform related to the porcine or bovine leukocyte 12-lipoxygenase. The DNA probe used in the Northern analysis (PL12lx) was 74% identical to the porcine leukocyte 12-lipoxygenase cDNA and might not be expected to hybridize to a similar human isoform, due to the high-stringency washing conditions employed. Alternatively, the 12-lipoxygenase mRNA, which is in relatively low abundance in HEL cells (estimate of 0.01–0.1% of total mRNA), may not have been detected by the RNA blot analysis in other tissues due to a lack of sensitivity. It remains possible that it might be detected by a more sensitive S1 nuclease assay or by PCR determination.

The availability of cDNA probes for the platelet/HEL 12-lipoxygenase will permit delineation of the structure–function relationships of the enzyme and a more precise exploration of the role of 12-lipoxygenase in human biology.

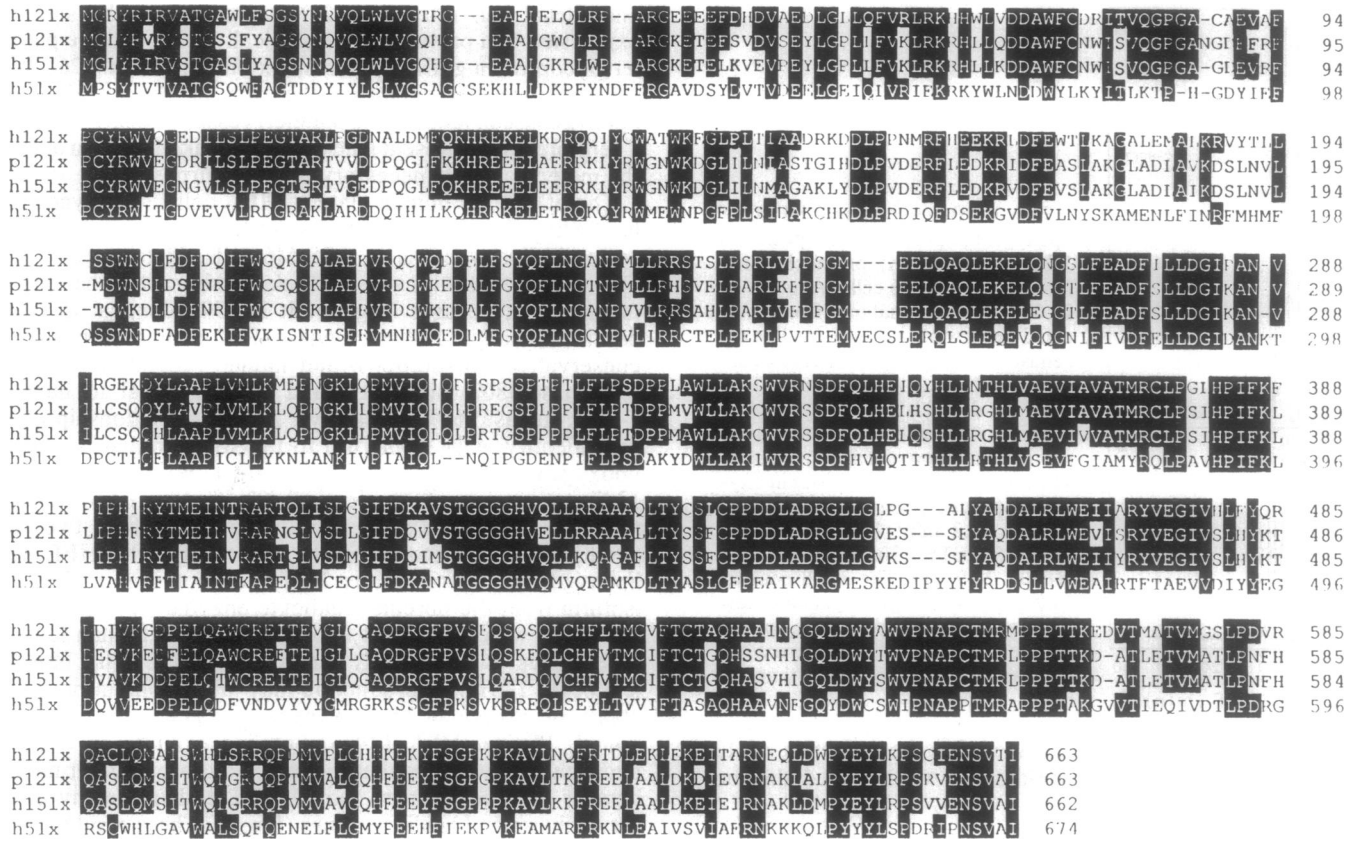

FIG. 5. Comparison of the amino acid sequences of lipoxygenases. Residues identical to human platelet/HEL 12-lipoxygenase are represented by white-on-black type. Numbering begins with the initiator methionine for each sequence. Dashes have been inserted to optimize sequence alignment. h12lx, human platelet/HEL 12-lipoxygenase; p12lx, porcine leukocyte 12-lipoxygenase; h15lx, human reticulocyte 15-lipoxygenase; h5lx, human 5-lipoxygenase.

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