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Cloning of the cDNA for human 5-lipoxygenase

(leukotrienes/transcriptional regulation/differentiation/HL60 cells)

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ABSTRACT **The enzyme 5-lipoxygenase (5-LO) catalyzes the first two reactions in the pathway leading to the formation of leukotrienes from arachidonic acid. Leukotrienes are potent arachidonic acid metabolites possessing biological activities that suggest a role in the pathophysiology of allergic and inflammatory diseases. To obtain structural information about 5-LO for use in developing anti-inflammatory chemotherapeutic agents, the enzyme was purified from human polymorphonuclear leukocytes and the amino acid sequences were determined for several cyanogen bromide-derived peptides. A cDNA clone encoding a 674-amino acid protein containing all of the derived peptide sequences was isolated from a dimethyl sulfoxide differentiated HL60 cell cDNA library. The mRNA encoding 5-LO was detected only in differentiated HL60 cells and not in the undifferentiated cell line, indicating that the expression of 5-LO in this cell line is transcriptionally regulated. In addition, the human protein displays some amino acid sequence homology with several lipases and significant homology with the partial sequences of soybean and reticulocyte lipoxygenases. Thus, 5-LO appears to be a member of a larger family of related enzymes.**

The enzyme 5-lipoxygenase (5-LO) catalyzes the first two reactions in the biosynthetic pathway leading to the formation of the leukotrienes from arachidonic acid. The first step is the addition of oxygen at carbon 5 of arachidonic acid, yielding (5*S*)-hydroperoxy-6,8,11,14-icosatetraenoic acid, which is transformed to leukotriene A_4 (5,6-oxido-7,9,11,14-icosatetraenoic acid) by dehydration in the second step (1–5). Leukotriene biosynthesis is known to occur in leukocytes after exposure to inflammatory or immunologic stimuli (1). The biological activities of these compounds, including leukocyte chemotaxis, airway constriction, increased vascular permeability, arteriolar constriction, and dilation of postcapillary venules, suggest a role for the leukotrienes in the pathophysiology of immediate hypersensitivity reactions and inflammation (1). Consequently, the regulation of 5-LO activity has recently become a subject of considerable interest.

The purification of 5-LO from human and porcine peripheral blood leukocytes (PMN), rat basophilic leukemia cells, and mouse mastocytoma cells has now been accomplished (3–7). The human enzyme requires Ca^{2+} for activity and is stimulated by ATP and a number of nondialyzable cellular components whose identity and function remain undefined (6). Despite these advances, very little is known about the structure of the enzyme or its regulation at the genetic level. In this paper, we report the cloning of the cDNA for 5-LO from differentiated HL60 cells and provide the complete amino acid sequence of the enzyme deduced from the cDNA sequence.¶

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MATERIALS AND METHODS

Protein Purification, Peptide Isolation, and Protein Sequencing. 5-LO was purified from human PMNs and the enzymatic activity was determined as described (2, 6). Protein determinations were performed by the method of Lowry (8). Proteins were analyzed by $NaDodSO_4$/PAGE as described by Laemmli (9). For amino acid sequence analysis, the final enzyme fraction of 5-LO (1.9 nmol) was precipitated with 15% trichloroacetic acid, rinsed with diethyl ether, and lyophilized. Peptides were prepared by treating 0.5 nmol of the purified protein with 0.4 mM CNBr in 70% formic acid at 23°C for 20 hr. After lyophilization, the samples were resuspended in 20 mM trifluoroacetic acid and the soluble peptides were separated by reversed-phase HPLC on a Vydac C-4 column eluted with a 10–70% acetonitrile gradient containing 20 mM trifluoroacetic acid. Amino-terminal amino acid sequence was determined for each purified peptide and for the intact protein with an Applied Biosystems (Foster City, CA) gas-phase sequenator as described (10, 11).

Cell Culture. HL60 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal bovine serum in slowly rotating roller bottles at 37°C. For differentiation, HL60 cells were diluted to 2×10^5 cells per ml and dimethyl sulfoxide and dexamethasone (Me_2SO/Dex) were added to 1.3% and 10 μM, respectively. Cells were harvested at various times by centrifugation at 500 × *g* for 10 min, washed once with phosphate-buffered saline, and frozen at −70°C. 5-LO activity in the cells was assayed as described (2).

RNA Isolation and Blotting. Total cellular RNA was isolated from HL60 cells by the guanidinium isothiocyanate/CsCl method (12). The poly(A)$^+$ RNA fraction was separated by chromatography on oligo(dT)-cellulose as described (13). For RNA blot analysis, 10 μg of total RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to nitrocellulose, and baked (14). The filter was prehybridized and hybridized in 5× standard saline citrate (SCC; 1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 6.8)/10× Denhardt's solution (1× Denhardt's solution is 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone)/50 mM $NaPO_4$, pH 6.8/0.1% $NaDodSO_4$/50 μg of denatured salmon sperm DNA per ml at 65°C. The double-stranded DNA probe was labeled by nick-translation with [α-^{32}P]dCTP and used at 10^6 cpm/ml in the hybridization. The filters were washed four times in 5×

Abbreviations: LO, lipoxygenase; PMN, polymorphonuclear leukocyte; Me_2SO/Dex, 1.3% dimethyl sulfoxide/10 μM dexamethasone.

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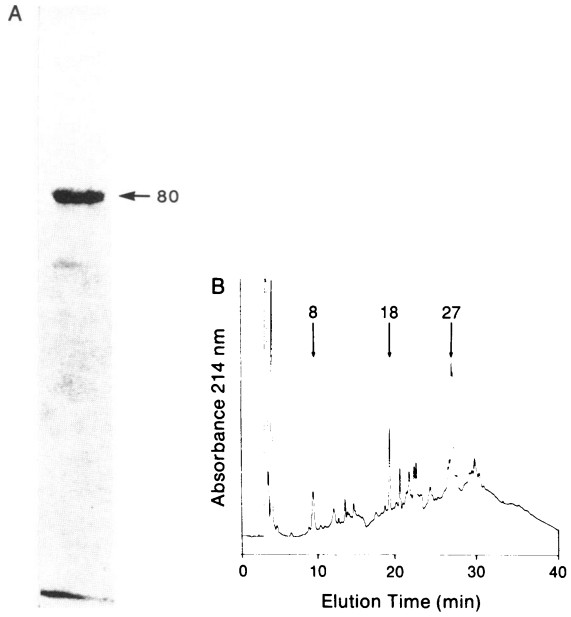
¶This sequence is being deposited in the EMBL/GenBank data base (Bolt, Beranek, and Newman Laboratories, Cambridge, MA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J03600).

SSC/0.1% $NaDodSO_4$ at 65°C, dried, and autoradiographed (14).

Oligonucleotide Synthesis. All oligonucleotides were synthesized using an Applied Biosystems model 380A DNA Synthesizer. Two overlapping complementary oligonucleotides, whose sequence was based on preferred codon usage, were synthesized corresponding to either the amino-terminal peptide (amino acids 12–22) or peptide-27 amino acid sequence. The oligonucleotide sequences corresponding to the amino-terminal peptide are 5′-pCAGTGGTTTGCTGGCACAGATGACTACATCTAC and 5′-pGTAGATGTAGTC. The oligonucleotide sequences corresponding to peptide 27 are 5′-pATGGCCCGGTTCCGGAAGAACCTGGAGGCCATTGTCTCTGTGATTGCTGAGCGGAACAAGAAG and 5′-pGGAGTTGGGGATCCGGTCAGGGGACAGGTAGTAGTATGGCAGCTGCTTCTTCTTGTTCCGCTC. For peptide 18, a pool of oligonucleotides was synthesized corresponding to all possible coding choices for amino acids 3–10 of peptide 18. The oligonucleotide sequences of the pool are 5′-pGA$^{A}_{G}$GA$^{A}_{G}$CA$^{T}_{C}$TT$^{T}_{C}$ATNGA$^{A}_{G}$AA$^{A}_{G}$CC.

cDNA Cloning. Double-stranded oligo(dT)-primed cDNA was synthesized from the HL60 cell poly(A)$^+$ RNA by a modification of the method of Gubler and Hoffman (15) as described (16). The cDNA was size-selected by agarose gel electrophoresis (14), ligated to λgt10 arms (17), and packaged *in vitro* (Gigapack, Stratagene, San Diego, CA). The library was plated on *Escherichia coli* LE392 and screened unamplified (14). The phage plaques were lifted onto nitrocellulose filters and processed for hybridization as described (14). The filters were prehybridized and hybridized in 5× SSC/10× Denhardt's solution/50 mM $NaPO_4$, pH 6.8/0.1% $NaDodSO_4$/50 μg of denatured salmon sperm DNA per ml/20% formamide (vol/vol) at 42°C. For hybridization, ^{32}P-labeled probes were used at 10^6 cpm/ml. The overlapping oligonucleotides were labeled with all four [α-^{32}P]dNTPs and Klenow DNA polymerase, the pool oligonucleotides were labeled with [γ-^{32}P]ATP and polynucleotide kinase, and double-stranded DNAs were labeled with [α-^{32}P]dCTP by nick-translation. Filters were washed four times in 5× SSC/0.1% $NaDodSO_4$ at temperatures ranging from 37°C to 55°C depending on the probe, dried, and autoradiographed (14). Hybridizing phage were purified and phage DNA was isolated as described (14). All other recombinant DNA techniques have been described (14).

DNA Sequence Analysis. The *Eco*RI inserts from the λgt10 5-LO clones were subcloned into both M13mp19 and pUC13 (18). The DNA sequence was determined by the dideoxynucleotide chain-termination method using either Klenow DNA polymerase or avian myeloblastosis virus reverse transcriptase and ^{35}S-labeled deoxyadenosine 5′-[α-thio]triphosphate (19, 20). The entire DNA sequence for both strands of each clone was determined.



C

N-TERMINUS: PSYTVTVATGSQWFAGTDDYIYLSLVGSA

PEPTIDE 8: VQRA

PEPTIDE 18: YPEEHFIEKPVKEA

PEPTIDE 27: ARFRKNLEAIVSVIAERNKKKQLPYYYLSPDRIPNS

FIG. 1. Purification and amino acid sequence determination of human PMN 5-LO. (*A*) The final enzyme fraction (10 μg) of 5-LO was electrophoresed on a 10% polyacrylamide gel containing $NaDodSO_4$ and silver-stained. Arrow indicates position and apparent molecular mass (in kDa) of the major polypeptide referred to in the text. (*B*) CNBr peptides were prepared from purified 5-LO as described. The peptides were resolved by reversed-phase HPLC using a Vydac C-4 column. A tracing of the absorbance profile at 214 nm of material eluting from the column is shown. The major peptide peaks are indicated by arrows. (*C*) Amino acid sequences obtained for the intact protein and the three CNBr-derived peptides indicated in *B* are shown. Single-letter amino acid code is used, beginning with the amino terminus of each peptide at the left. The first amino acid shown in all cases was determined in the first cycle of the sequenator and should represent the amino-terminal amino acid of that peptide.

RESULTS

5-LO Protein Characterization. 5-LO was purified from human PMNs as described (2, 6). The final enzyme preparation, which was ≈85% pure as assessed by $NaDodSO_4$/PAGE (Fig. 1*A*), contained a major polypeptide with an apparent molecular mass of 80 kDa and a minor component of 63 kDa. The size of the 80-kDa band is consistent with the previously published estimates for the rat, porcine, and human enzymes (3–7). To obtain peptide sequence for 5-LO, the protein was subjected to chemical cleavage with CNBr. The soluble peptides resulting from the cleavage were resolved by reversed-phase HPLC and purified (Fig. 1*B*). The three fractions containing the most UV absorbing material and the intact purified protein were subjected to amino acid sequence analysis yielding the sequences shown in Fig. 1*C*. It should be noted that the amino acid sequence determined for the amino terminus of intact human

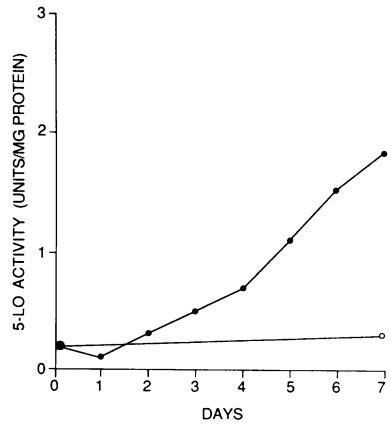


FIG. 2. Time course for induction of 5-LO activity in HL60 cells after differentiation. HL60 cells were seeded at 2 × 10^5 cells per ml in the presence (●) or absence (○) of Me_2SO/Dex as described. The cells were harvested at the times indicated and assayed for 5-LO activity. One unit of 5-LO activity is the amount of enzyme that will produce 1 nmol of (5*S*)-hydroperoxy-6,8,11,14-icosatetraenoic acid in 10 min at 37°C using 100 μM arachidonic acid as the substrate.

5-LO is identical to the first 25 amino acids reported for the purified rat 5-LO (5). That peptide 27 is derived from the 80-kDa polypeptide is demonstrated by the observation that an antisera directed against a peptide with the same sequence reacts with the 80-kDa and not the 63-kDa polypeptide by immunoblotting (data not shown).

HL60 Cell Expression of 5-LO Activity. Although an adequate source of protein, circulating PMNs proved to be a poor source of mRNA for use in cDNA library construction. Consequently, alternative cell lines were examined for 5-LO expression. Upon exposure to dimethyl sulfoxide, the human promyelocytic leukemia cell line HL60 can be induced to differentiate into cells that closely resemble mature granulocytes and that are capable of synthesizing leukotrienes (21–23). HL60 cells were seeded in the presence or absence of Me_2SO/Dex, allowed to grow, and tested for 5-LO activity. As shown in Fig. 2, little 5-LO activity was detected in untreated HL60 cells. However, cells grown for 7 days in the presence of Me_2SO/Dex contained 6–8 times the amount of 5-LO activity found in the untreated cells. The increase in 5-LO activity was continuous and linear throughout the time course of the experiment.

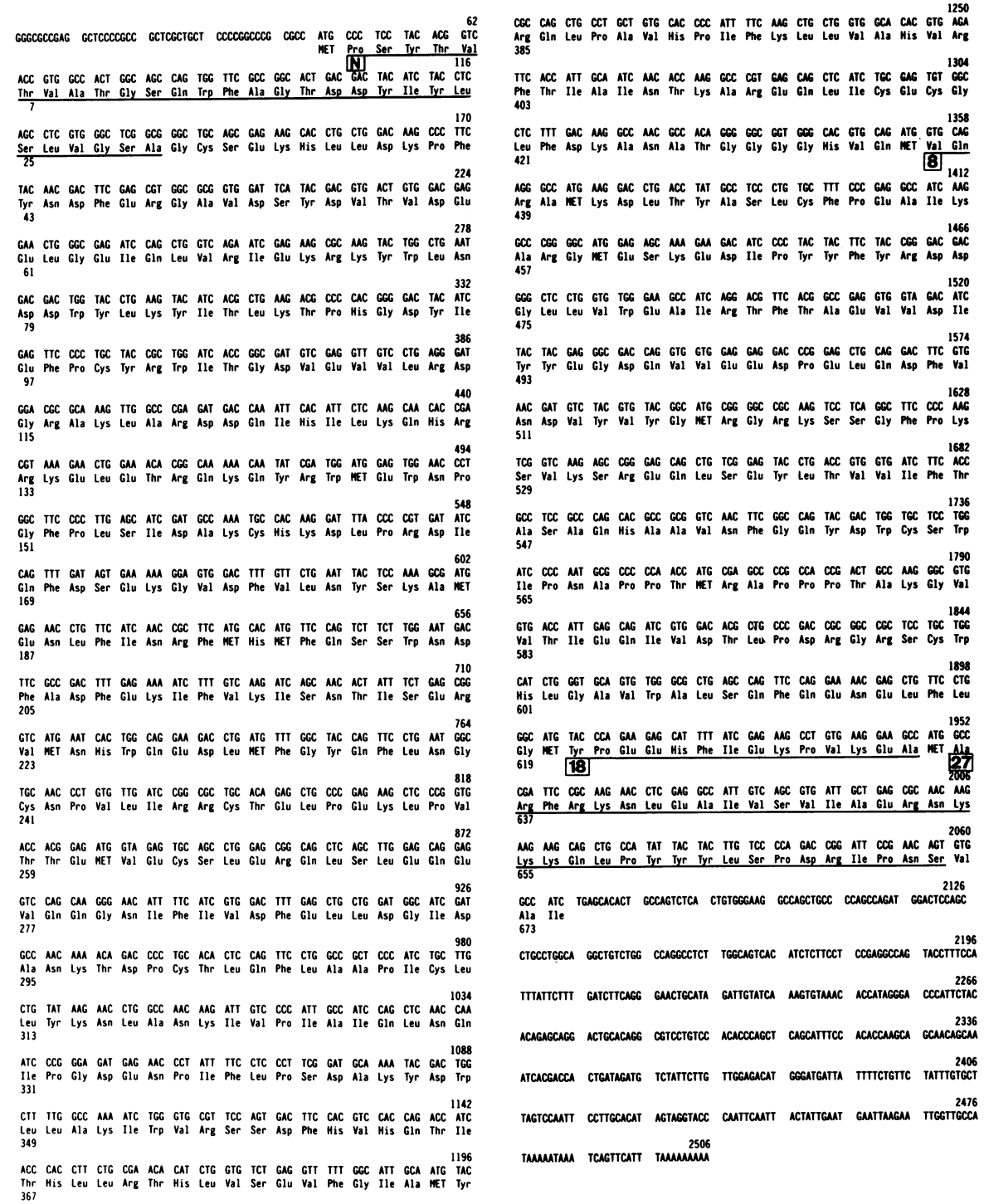


FIG. 3. DNA sequence for the *Eco*RI insert of λ5LO-6 is shown. The amino acids predicted to be encoded by the DNA sequence are presented beginning at the first ATG contained within the clone. The positions of the derived peptide sequences shown in Fig. 1*C* are underlined and numbered accordingly. The nucleotides are numbered on the right of each line and the amino acids are numbered on the left.

cDNA Cloning. Poly(A)$^+$ RNA was isolated from HL60 cells grown for 5 days in the presence of Me_2SO/Dex and used to construct a cDNA library in λgt10. Oligonucleotide probes were synthesized corresponding to the amino-terminal peptide sequence and the sequences of peptides 18 and 27. These oligonucleotides were used to screen 2×10^6 recombinants from the HL60 cDNA library. A total of 35 clones were isolated that hybridized to probes for both peptide-18 and -27 sequences. No clones were isolated that hybridized to the oligonucleotides corresponding to the amino-terminal peptide sequence. Restriction analysis of the hybridizing clones revealed that all contained inserts ranging from 1.0 to 1.3 kilobases whose restriction maps overlapped. The inserts from the three longest clones (λ5LO-10, λ5LO-28, and λ5LO-30) were sequenced and shown to contain identical overlapping DNA sequences. The cDNA sequence contained an open reading frame capable of encoding 211 amino acids, including the amino acid sequences of peptides 18 and 27, but not of the amino-terminal peptide or peptide 8. The DNA sequences encoding peptides 18 and 27 were at the extreme 3′ end of the open reading frame. All clones contained a stretch of poly(A) residues 428 bases 3′ of the open reading frame. These clones, therefore, probably correspond to the 3′ end of the 5-LO mRNA.

To obtain full-length cDNA clones for 5-LO another cDNA library was made using poly(A)$^+$ RNA from 7-day differentiated HL60 cells. The double-stranded cDNA was size-selected for those cDNAs >2.2 kilobases prior to ligation into the λgt10 vector arms. This library contained 500,000 recombinants, which were screened unamplified with both a restriction fragment from λ5LO-30 (see Fig. 3, nucleotides 1430–1740) and with the oligonucleotides corresponding to the amino-terminal peptide. Of the 32 clones that hybridized to the λ5LO-30 probe, 8 also hybridized to the probe for the amino-terminal sequence. These 8 clones contained inserts between 2.4 and 2.6 kilobases long and had restriction maps that overlapped the clones corresponding to the 3′ end of the 5-LO mRNA. DNA sequencing of two of the clones (λ5LO-4 and λ5LO-6) revealed that both contained an identical open reading frame of 674 amino acids following the first ATG in the sequence (Fig. 3). This open reading frame would encode a polypeptide of 78 kDa, containing all of the 5-LO-derived peptide sequences (see Figs. 1*C* and 3). The first ATG contained in the cDNA clones immediately precedes the DNA sequence that encodes the amino-terminal peptide sequence derived from the mature protein. The peptide sequence that would be encoded by the DNA sequence preceding this ATG does not resemble any known leader or signal sequence. In addition, the DNA sequence surrounding this ATG is similar to the consensus sequence for eukaryotic initiation codons (24). Therefore, while the possibility of upstream initiation at an ATG not contained in this cDNA cannot be ruled out, it is likely that this codon serves as the start site for translation of 5-LO. As in the partial 5-LO clones, the termination codon is followed by 428 bases of 3′ untranslated sequence. All of the clones contain a stretch of poly(A) residues at their 3′ end, which is preceded by the consensus polyadenylylation signal sequence AATAAA.

Expression of 5-LO Specific RNA in HL60 Cells. The size of the mRNA corresponding to the cDNA clones was determined by RNA blot analysis using total RNA from differentiated and undifferentiated HL60 cells (Fig. 4). The cDNA hybridized to a single RNA species of 2700 nucleotides that was detected in the differentiated cell RNA and not in the undifferentiated cell RNA. The detection of mRNA homologous to λ5LO-30 only in cells expressing 5-LO activity indicated that the induction of 5-LO activity in HL60 was regulated at the transcriptional level. In addition, the size of the mRNA suggests that the 5-LO clones that were obtained represent almost the entire message.

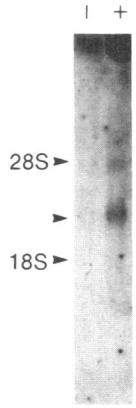


FIG. 4. RNA blot analysis of 5-LO mRNA expression in differentiated and undifferentiated HL60 cells. Total cellular RNA was isolated from HL60 cells grown in the presence (+) or absence (−) of Me_2SO/Dex for 5 days. The RNA (10 μg) was electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to nitrocellulose, and processed as described. The *Eco*RI insert from λ5LO-30, which encompasses the 3′ third (nucleotides 1433–2506 in Fig. 3) of the 5-LO coding region, was nick-translated and used as the hybridization probe. The positions of 28S and 18S ribosomal RNA are indicated. Arrowhead designates the location of the 2700-nucleotide species referred to in the text.

DISCUSSION

This report describes the cloning of the cDNA for human 5-LO based on partial protein sequence. The availability of a clone for 5-LO should prove useful for studying the structure of the enzyme and the regulation of 5-LO gene expression. Expression of 5-LO is restricted to leukocytes and a few other tissues. Stimulation of cells containing 5-LO with agents that increase Ca^{2+} activates the enzyme and allows the cells to produce leukotrienes, provided that free arachidonic acid is available. In HL60 cells, the ability to synthesize leukotrienes in response to Ca^{2+} ionophores requires that the cells be matured to granulocytes. The induction of leukotriene synthetic capability appears to involve an increase in both 5-LO mRNA and 5-LO protein. Thus, it is suggested that 5-LO gene expression is induced when the cells undergo differentiation. This system could, therefore, prove useful for defining the factors that control 5-LO gene regulation as well as granulocyte differentiation.

The predicted amino acid sequence of 5-LO was examined by the methods of Kyte and Doolittle (25) and Hopp and Woods (26) to determine the distribution of hydrophobic residues in the protein. While the central portion of the sequence is moderately hydrophobic, the protein is overall hydrophilic (average hydrophobicity, −0.28). There are no signal sequences or membrane-spanning domains predicted from the sequence, in agreement with the fact that 5-LO is only peripherally associated with the membrane when activated with Ca^{2+} (27). Interestingly, we note the presence of a sequence between amino acids 368 and 382 (His-Leu-Leu-Arg-Thr-His-Leu-Val-Ser-Glu-Val-Phe-Gly-Ile-Ala) that is related to the interface-binding domain of the human lipoprotein lipase (His-Leu-Leu-Gly-Tyr-Ser-Leu-Gly-Ala-His-Ala-Ala-Gly-Ile-Ala) (28) and the rat hepatic lipase (His-Leu-Ile-Gly-Tyr-Ser-Leu-Gly-Ala-His-Val-Ser-Gly-Phe-Ala) (29). As in the lipases, this peptide segment is localized within a region of overall hydrophobicity. The presence of this sequence suggests that the mechanism for 5-LO enzyme–substrate interaction might be similar to that of the lipases. As 5-LO activity is affected by Ca^{2+} and ATP, the amino acid sequence was examined for the presence of consensus binding sites for these cofactors. No strong homology was observed in the sequence to any ATP binding sites. Two regions of the sequence (amino acids 16–28 and 488–512) exhibited weak homology to the consensus Ca^{2+}-binding sites from lipocortin (30, 31) and calmodulin (32), respectively. The position of the actual Ca^{2+}-binding site will have to be determined by direct biochemical and genetic analysis, however.

Comparison of the complete amino acid sequence for human 5-LO with the sequences for other cloned lipoxygenases revealed a significant amount of homology to the soybean *Lox1* gene product and the rabbit reticulocyte

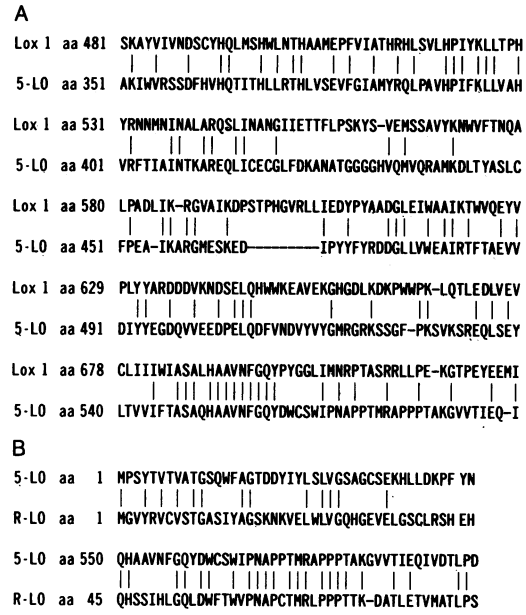


FIG. 5. Comparison of the amino acid sequence of 5-LO with those of (*A*) the soybean Lox1 protein and (*B*) the rabbit reticulocyte LO. All sequences are aligned for maximum homology with identical matches indicated. Only those regions of each protein exhibiting significant amino acid homology are shown. Each amino acid sequence is numbered individually on the left of each line. Amino acids are identified by the single-letter code.

15-LO (Fig. 5). The Lox1 protein catalyzes an enzymatic reaction similar to 5-LO, but with differences in substrate preference and positional specificity (33). The greatest amino acid homology was observed between the central portions of both 5-LO and the Lox1 protein. In one region of 5-LO, 12 of 13 amino acids (including one serine, one histidine, and one tyrosine, amino acids likely to be present within the active site of the enzyme) are shared with the Lox1 protein. A comparison of the hydrophobicity profiles (25, 26) of the two proteins indicated a similar distribution of hydrophobic and hydrophilic residues throughout both proteins. Likewise, both proteins had similar overall secondary structures as predicted by the method of Chou and Fasman (34). The high degree of sequence conservation between an enzyme from plants and one from animals suggests that the conserved regions may play a role in enzymatic functions. The amino-terminal 88 amino acids of the rabbit reticulocyte 15-LO have been reported (35). While it is difficult to draw functional conclusions from the limited amount of sequence data, two regions of significant similarity were observed. The first region of similarity is at amino acids 1–34 of 15-LO and amino acids 1–34 of 5-LO, while the second is at amino acids 550–594 of 5-LO and amino acids 45–88 of 15-LO. The division of the similar regions in 5-LO may reflect structural or functional differences between the two enzymes. Thus, all of these enzymes that catalyze similar reactions may have diverged from a common ancestral gene product and represent members of a large family of related proteins. Taking the structural similarities into account, it should be possible to use molecular modeling studies combined with biochemical and genetic analysis to determine the structure–function relationship of 5-LO and design more effective chemotherapeutic agents that use 5-LO as a target.

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