Cloning of the cDNA for human 12-lipoxygenase  
(arachidonic acid/platelets/human erythroleukemia cells/DNA sequence/expression)  
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ABSTRACT A full-length cDNA clone encoding 12-lipoxygenase (arachidonate:oxygen 12-oxidoreductase, EC 1.13.11.31) was isolated from a human platelet cDNA library by using a cDNA for human reticulocyte 15-lipoxygenase as a probe for the initial screening. The cDNA had an open reading frame encoding 662 amino acid residues with a calculated molecular weight of 75,590. Three independent clones revealed minor heterogeneities in their DNA sequences. Thus, in three positions of the deduced amino acid sequence, there is a choice between two different amino acids. The deduced sequence from the clone pIF3 showed 65% identity with human reticulocyte 15-lipoxygenase and 42% identity with human leucocyte 5-lipoxygenase. The 12-lipoxygenase cDNA recognized a 3.0-kilobase mRNA species in platelets and human erythroleukemia cells (HEL cells). Phorbol 12-tetradecanoyl 13-acetate induced megakaryocytic differentiation of HEL cells and 12-lipoxygenase activity and increased mRNA for 12-lipoxygenase. The identity of the cloned 12-lipoxygenase was assured by expression in a mammalian cell line (COS cells). Human platelet 12-lipoxygenase has been difficult to purify to homogeneity. The cloning of this cDNA will increase the possibilities to elucidate the structure and function of this enzyme.

The enzyme 12-lipoxygenase (arachidonate:oxygen 12-oxidoreductase, EC 1.13.11.31) catalyzes the formation of (12S)-12-hydroperoxy-(5Z,8Z,10E,14Z)-5,8,10,14-eicosatetraenoic acid (12-HPETE), which is further reduced to the corresponding hydroxy fatty acid, (12S)-12-hydroxy-(5Z,8Z,10E,14Z)-5,8,10,14-eicosatetraenoic acid (12-HETE). This enzyme has been found in various mammalian tissues: platelets (1, 2), porcine and bovine leukocytes (3–5), murine eosinophils (6), bovine tracheal cells (7), and porcine pituitary cells (8). Many biological functions of 12-lipoxygenase metabolites have been reported for various tissues, such as a chemotacticant in rat aortic smooth muscle cells (9), a neurotransmitter in Aplysia neuronal cells (10), and an activator of a glycoprotein IIb/IIIa-like receptor in tumor cells (11). It was proposed that 12-lipoxygenases isolated from various tissues are heterogeneous, based on biochemical and immunological studies (2, 4, 7, 12).

The amino acid sequences of mammalian 5- and 15-lipoxygenases were deduced from their cDNAs (13–17), and recently cDNA for porcine 12-lipoxygenase was isolated (18). Among these lipoxygenases, there is a certain homology in the amino acid sequences. Especially, porcine leukocyte 12-lipoxygenase exhibited 86% identity with human reticulocyte 15-lipoxygenase (18).

Human platelet 12-lipoxygenase has not yet been purified to homogeneity. In this study, we isolated cDNA for this enzyme by using a cDNA of human reticulocyte 15-lipoxygenase as the initial hybridization probe. We found that phorbol 12-tetradecanoyl 13-acetate (TPA) induced 12-lipoxygenase activity and 12-lipoxygenase mRNA in human erythroleukemia cells (HEL cells). The 12-lipoxygenase activity was expressed in a mammalian cell line (COS cells, a monkey kidney cell line).

MATERIALS AND METHODS  

cDNA Library. To obtain RNA for construction of a cDNA library, 25 bags of buffy coat from healthy donors were collected from a local blood center. After removal of erythrocytes by dextran sedimentation and ammonium chloride lysis, buffy coat (containing about 2 × 10^10 leukocytes and 10^10 platelets) and platelets (containing about 3 × 10^11 platelets and <10^9 leukocytes) were purified by centrifugations. Total cellular RNAs were isolated by acid guanidinium isothiocyanate/phenol/chloroform extraction (19), and the poly(A)^+ RNA fractions were separated by oligo(dT)-cellulose chromatography (20). Double-stranded cDNA was prepared by using a Pharmacia cDNA synthesis kit with oligo(dt) cellulose chromatography (20). The cDNA was size-selected on a 1% agarose gel to enrich cDNA longer than 2.0 kilobases (kb) and was ligated to the phage λ ZAP II vector (Stratagene) with an EcoRI/Not 1 adaptor.

CDNA of Human 15-Lipoxygenase. Two oligonucleotides (5'-TTCTATGCGCAAGATGCCTGCG-3', 5'-GCAGCAGCTCTCTCCGGACTT-3') were synthesized according to the sequence data published for human 15-lipoxygenase (16). Utilizing these two oligonucleotides as polymerization primers and the double-stranded cDNA from buffy coat as template, a cDNA fragment was obtained by a polymerase chain reaction (21). This cDNA was inserted into the plasmid vector pUC19 (digested with BamHI and HindIII) and amplified in Escherichia coli. Digestion with EcoRI and HindIII gave a fragment (431 bp) that was purified on a 1.2% agarose gel and 32P-labeled with an oligonucleotide labeling kit (Pharmacia). The cDNA was cloned into the BamHI site of the lambda ZAP II vector containing the cDNA inserts.

Screening of cDNA Libraries. Transfected E. coli (XL-Blue) were grown on 90-mm plastic dishes at 5000 plaques per dish. Plaques were transferred to nitrocellulose filters (Millipore HATF), which were treated as described (20). The filters were prehybridized in a solution containing 40% (vol/vol) formamide, 5× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate), 5× Denhardt’s solution (1× 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.1% NaDODSO4, and 100 μg of denatured salmon sperm DNA per ml at 42°C for 4 hr. 32P-labeled probe was added, and hybridization was continued overnight. Filters were washed with 0.1× SSC, 0.1% SDS at 65°C for 15 min. Filters were then washed 2× in 0.1× SSC, 0.1% SDS at 65°C for 15 min and then placed in autoradiographic film (DuPont) for 2 days.

Abbreviations: 12-HPETE, (12S)-12-hydroperoxy-(5Z,8Z,10E,14Z)-5,8,10,14-eicosatetraenoic acid; HEL, human erythroleukemia cells; TPA, phorbol 12-tetradecanoyl 13-acetate.

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This sequence has been deposited in the GenBank data base (accession no. M38792).
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washed twice at room temperature for 15 min with 2× SSC/0.1% NaDodSO4 and then twice with 0.2× SSC/0.1% NaDodSO4 at 55°C for 30 min. The filters were dried and exposed to Fuji x-ray film with an intensifying screen at −70°C for 12–16 hr.

DNA Sequence Analysis. The EcoRI inserts of positive phage clones were rescued into the pBluescript plasmid by using a helper phage according to the manufacturer’s protocol (Stratagene). Appropriate restriction fragments were subcloned into phage vectors M13 mp18 or mp19, and DNA sequencing was carried out by the dideoxy chain-termination method (22) with phage T7 DNA polymerase (Pharmacia). In some cases, 7-deaza-dGTP was used to obtain clear sequencing of G+C-rich regions. Synthetic oligonucleotide primers were also utilized to determine the entire sequence for both strands. Sequence data were analyzed and compared by using software from the University of Wisconsin Genetics Computer Group.

Differentiation and 12-Lipoxigenase Activity of HEL Cells. HEL cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum. HEL cells are known to have numerous megakaryocytic markers that are markedly enhanced after the addition of TPA (23). For differentiation, HEL cells were diluted to 1.5 × 10⁶ cells per ml, and TPA was added to 80 or 160 nM. The cells started to attach to the Petri dish 5 min after addition of TPA, and within 2–3 days marked morphological changes (increased cell size and larger and lobulated nuclei) were apparent (24). Cells were harvested at various times by centrifugation at 500 × g for 10 min, washed once with phosphate-buffered saline, and resuspended in 0.05 M Tris-HCl/0.1 M NaCl/2 mM CaCl2/2 mM glutathione, pH 7.4, at a concentration of 5–20 × 10⁶ cells per ml. Cell suspensions (routinely 0.5 ml) were preincubated at 37°C for 2 min and arachidonic acid (160 μM) and calcium ionophore A23187 (2 μg/ml) were added. As an activator, 13-hydroperoxy-9,11-octadecadienoic acid (5 μM) was also added (25). After 10 min, the incubation was terminated by the addition of 2 volumes of stop solution (acetonitrile/methanol/acidic acid, 350:150:3, vol/vol). After centrifugation, the supernatant was applied to a mini-ODS silica column (Chromabond C18; Düren, F.R.G.), and the lipid fraction was recovered with 2 ml of methanol. After evaporation, an aliquot of the sample was analyzed on a reversed-phase HPLC column (ODS 100-5, 250 × 4.6 mm, Nucleosil), with solvent system G (acetonitrile/methanol/water/acidic acid, 350:150:250:1, vol/vol) at a flow rate of 1.5 ml/min. The UV monitor was set at 235 nm. The activator, 13-hydroperoxy-9,11-octadecadienoic acid, was usually recovered as the reduced form (13-hydroxy-9,11-octadecadienoic acid) with a recovery rate of 85–95%. 12-Lipoxigenase activity was calculated by the peak area ratio of 12-HETE to 13-hydroxy-9,11-octadecadienoic acid. Standard 12-HETE and 13-hydroxy-9,11-octadecadienoic acid were eluted at 16.8 min and 13.7 min, respectively.

RNA Blot Analysis. Poly(A)+ RNA was purified from undifferentiated HEL cells, differentiated HEL cells, platelets, and the leukocyte-enriched fraction from buffy coat (13). For blot analysis, 1 μg of each sample was electrophoresed on a 1% agarose gel containing 0.7% formaldehyde, transferred to nitrocellulose filter, and baked (20). The Not I insert from the clone pLT3 (see Results and Discussion) was radiolabeled with [α-32P]dCTP by using random hexamers (oligo-labeling kit, Pharmacia) and was used as hybridization probe. The filter was prehybridized, hybridized, washed, and autoradiographed as described for the screening of the cDNA library.

COS Cell Expression. The COS cell expression vector CDMS8 (26) and E. coli MC1061/p3 were provided by Brian Seed, Massachusetts General Hospital, Boston. The Not I fragment of the clone pLT3 containing the 12-lipoxigenase cDNA was ligated to CDMS8 (opened with BstXI) by using a Not I/BstXI adaptor prepared by annealing two oligonucleotides (5'-CTGGTACCGC and 5'-GGCCGCGTACGACA). This gave the plasmid CDMS8pLT3, which was amplified in E. coli MC1061/p3. The plasmid DNA was purified by an alkaline lysis method combined with a Qiagen-tip (Diagen, Dusseldorf, F.R.G.).

COS cells (a monkey kidney cell line) were maintained in a Dulbecco's modified Eagles's medium (DMEM, Nordvacc, Skärholmen, Sweden) supplemented with 10% (vol/vol) fetal bovine serum. COS cells (8 × 10⁵ cells) at 25% confluency in 100-mm dishes were transfected in 3.75 ml of OPri-MEM I medium (GIBCO) containing 400 μg of DEAE-dextran (Pharmacia) per ml, 100 μM chloroquine diphosphate, and 2 μg of the purified DNA (27). After incubation for 90 min, the medium was removed, and the transfected cells were incubated in DMEM/10% fetal bovine serum for 48–72 hr to allow for expression. The cells were detached by incubation in phosphate-buffered saline with 5 mM EDTA, pooled, and resuspended in ice-cold phosphate-buffered sa-

![Fig. 1. Restriction map and sequencing strategy of human platelet 12-lipoxigenase cDNA.](image)

The protein coding region is indicated by an open bar. The closed circles indicate restriction sites, and the open circles indicate oligonucleotide primers. Direction and extent of sequence determination are indicated by arrows. The broken line of bcM2 (region upstream of "a") indicates the putative intron sequence, and at position "b", 4 bp are missing (see Results and Discussion). The 12-lipoxigenase cDNA sequence (Fig. 2) was deduced from clones pLT3 (nucleotides from -33 to 2289) and bcM1 (nucleotides 2290–2302).
The 12-lipoxygenase activity of the cells was assayed as described above, except that the ODS column extraction was omitted.

RESULTS AND DISCUSSION

Isolation of cDNA Clones. In the initial screening of 4 × 10^5 plaques from a buffy coat-derived cDNA library by using the reticulocyte 15-lipoxygenase cDNA as hybridization probe, two weakly positive clones were isolated (bcM1 and bcM2). The clone bcM1 had about 1400 bp of open reading frame, a termination codon (TAG), and a polyadenylation code (AAATAA).

The deduced amino acid sequence of bcM1 revealed about 60% identity with the corresponding sequence of human reticulocyte 15-lipoxygenase and 40% with human leukocyte 5-lipoxygenase and contained five conserved histidine residues that are characteristic for the lipoxygenase family (13–18). Thus, the clone bcM1 was supposed to encode a lipoxygenase other than 5-lipoxygenase or 15-lipoxygenase. The clone bcM2 was quite similar to bcM1 but had a different 5'-end sequence (94 bp starting with the position "a" in Fig. 1, nucleotide 645 in Fig. 2). Thus, the sequence 5'-CGCGCTGAAGAAGAAGCAGGGTTCA-3' of bcM1 was changed to 5'-CGCGCTGAAGAAGAAGCAGGGTTCA-3' in bcM2. Also, 4 bp (GCCG, positions 1249–1252) were missing in the middle of the open reading frame of bcM1 (at position "b" in Fig. 1). The analysis of the genes for human 5-lipoxygenase and rabbit 15-lipoxygenase indicated that these two lipoxygenases have similar splicing structures; alignment of the sequences revealed well-matched exon-intron relations.

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KCSCCTCCTCTTGAGCTGCGCTGCTGGCGAGCAGCGCCACAGACACATGGGAATGAGTGTGACTATGTTCCZMAWCTTTATGGACAC

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Fig. 2. Nucleotide sequence of the cDNA for human platelet 12-lipoxygenase and the deduced amino acid sequence. Nucleotide residues are numbered from 5′ to 3′ with the first residue at the ATG codon (encoding the initiating methionine). The deduced amino acid sequence is displayed below the nucleotide sequence in the three-letter code. Underlines indicate the initiation codon ATG, the termination codon TGA, and the polyadenylation signal AAATAA. Some heterogeneities were observed among the clones bcM1, bcM2, and pFT3. Alternative DNA sequences are shown in parentheses (see also Table 1).
tionships (28). Comparison of the DNA sequences of rabbit 15-lipoxygenase, human 5-lipoxygenase, and human 12-lipoxygenase indicated that both positions “a” and “b” of the clone bcM2 are located just at the fifth and ninth intron-exon junctions, respectively. Thus, it is possible that the changes at positions “a” and “b” might have resulted from splicing divergencies. However, these divergencies could also be due to cloning artifacts. The clone bcM2 had a longer 3′ untranslated region (0.82 kb), which indicated the presence of an alternative polyadenylation signal in the gene for 12-lipoxygenase.

To obtain a full-length clone, a human platelet library was prepared. About 2 × 10^6 clones were screened by using the radiolabeled PstI fragment (552 bp) of bcM1 as probe. Three positive clones were isolated; the clone pIT3 had the longest DNA insert (2.3 kb) and seemed to encode a full-length amino acid sequence based on the homology to other lipoxygenases.

Nucleotide Sequence of cDNA and Deduced Amino Acid Sequence for 12-Lipoxygenase. The cDNA sequence corresponding to human 12-lipoxygenase was primarily obtained from clone pIT3 (nucleotides from −33 to 2289), and to some extent from clone bcM1 (nucleotides from 2290 to 2302). Codon ATG at nucleotides 1–3 was designated as the translation initiation codon not only because of a homology to other lipoxygenases but because the upstream region of this ATG has similarity to the eukaryotic initiation site. Thus in pIT3, guanosine was present at position −3 and cytidine was present at positions −1, −2, and −4 in good agreement with the consensus sequence in which a purine is present in position −3 and cytidine is predominant at positions −1, −2, −4, and −5 (29). A termination codon TGA (nucleotides 1990–1992) was followed by a 3′ untranslated region of 310 bp. The nucleotide sequence AATAAA (polyadenylation signal) was present at nucleotide 2282–2287. The open reading frame encoded a protein of 662 amino acids, excluding the

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<th>Position of heterogeneity</th>
<th>Codons and corresponding AA of clones</th>
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<td>bcM1</td>
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<td>254</td>
<td>TCG</td>
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<td>260</td>
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<td>321</td>
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AA, amino acid.

About 18% of the nucleotides in the cDNA sequences were obtained between the clones bcM1, bcM2, and pIT3 at five positions (Table 1). Three of them caused changes of the deduced amino acid residues.

A sequence, His-(Xaa)_4-His-(Xaa)_4-His-(Xaa)_17-His-(Xaa)_4-His, has been found in many lipoxygenases and has been proposed to be the putative iron-binding domain (30). We also found this domain at amino acid residues 354–391 in human 12-lipoxygenase. Human 12-lipoxygenase exhibited 65% identity and 80% similarity to human reticulocyte 5-lipoxygenase, 42% identity and 62% similarity to human 5-lipoxygenase, and 66% similarity and 78% identity to porcine 12-lipoxygenase (GAP program, University of Wisconsin, Genetic Computer Group). This supports the previous speculation that 12-lipoxygenase is evolutionary closer to 15-lipoxygenase than to 5-lipoxygenase (18).

12-Lipoxygenase in HEL Cells. Upon exposure to TPA, the HEL cells can be induced to differentiate into megakaryocytic cells (23, 24). HEL cells were seeded in the presence or absence of TPA and tested for 12-lipoxygenase activity. Little activity was detected in untreated HEL cells (Fig. 3). After 2 days of incubation in the presence of TPA, the 12-lipoxygenase activity in the cells increased 3–4 times

![Fig. 3](image-url)  
**Fig. 3.** Time course for induction of 12-lipoxygenase (12-LO) activity in HEL cells after differentiation. HEL cells were seeded at 1.5 × 10^6 cells per ml in the presence (b) or absence (c) of TPA at a concentration of 160 nM. The cells were harvested at the times indicated and assayed for 12-lipoxygenase activity as described.

![Fig. 4](image-url)  
**Fig. 4.** RNA blot analysis of 12-lipoxygenase mRNA expression in HEL cells, platelets, and leukocytes. Poly(A)^+^ RNAs (1.0 μg) from undifferentiated HEL cells (lane 1), differentiated HEL cells (80 nM TPA for 3 days) (lane 2), platelets (lane 3), and leukocytes (lane 4) were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to nitrocellulose, and processed as described. The Not I insert from clone pIT3, which encompasses the full coding region of 12-lipoxygenase, was radiolabeled by using random hexamers and was used as hybridization probe. The positions of 28S and 18S ribosomal RNAs are indicated.

![Fig. 5](image-url)  
**Fig. 5.** Structure of the plasmid CDM8pIT3 used for the expression of human platelet 12-lipoxygenase in COS cells. CMV/T7, cytomegalovirus/phage T7 RNA polymerase promoter; splice + An, splice and polyadenylation signals from plasmid pSV2; Py ori, polyomavirus origin of replication; SV40 ori, simian virus 40 origin of replication; M13 ori, M13 origin of replication; SupF, supF gene.
compared with the untreated cells. This increase remained throughout the time course of the experiment.

**RNA Blot Analysis of 12-Lipoxygenase mRNA.** The 12-lipoxygenase cDNA probe recognized a 3.0-kb mRNA species in HEL cells (Fig. 4). This mRNA became more abundant in the differentiated HEL cells. However, human platelets seemed to have two bands. One band was 3.0 kb and the other 2.8 kb. It is uncertain if this smaller-sized band indicates the presence of another species of 12-lipoxygenase mRNA or if it is caused by degradation of mRNA in platelets. In addition, no positive band was observed in leukocyte RNA.

**Expression of Cloned 12-Lipoxygenase in a Mammalian Cell.** 12-Lipoxygenase activity was expressed in a monkey kidney cell line (COS cells). The cDNA of the clone pIT3 was introduced into the expression vector CMV8, giving CMV8pIT3 (Fig. 5), which was transfected into COS cells. Enzyme activity was not detected in the nontransfected COS cells or the cells transfected with wild-type CMV8. Two days after transfection, 12-lipoxygenase activity of the COS cells could be detected (3.5 and 9.4 ng of 12-HETE per 10^6 cells in two independent experiments), and at day 3 the activity was increased (13.8 and 16.4 ng of 12-HETE per 10^6 cells). HPLC chromatograms of the products obtained from incubations of the intact COS cells with arachidonic acid are shown in Fig. 6.

The availability of a cDNA for human platelet 12-lipoxygenase will facilitate further studies regarding the structure and function of this enzyme.

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