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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 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 plT3 showed 65% identity with human reticulocyte 15-lipoxygenase and 42% identity with human leukocyte 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 (12*S*)-12-hydroperoxy-(5*Z*,8*Z*,10*E*,14*Z*)-5,8,10,14-eicosatetraenoic acid (12-HPETE), which is further reduced to the corresponding hydroxy fatty acid, (12*S*)-12-hydroxy-(5*Z*,8*Z*,10*E*,14*Z*)-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 chemoattractant 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 $<10^8$ 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)$_{12-18}$ primer. 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 *Eco*RI/*Not* I adaptor.

cDNA of Human 15-Lipoxygenase. Two oligonucleotides (5′-TTCTATGCCCAAGATGCGCTGCG-3′, 5′-GCAGCCAGCTCCTCCCTGAACTT-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). The cDNA thus obtained encoded about one-fourth of the amino acid sequence of the 15-lipoxygenase (close to the C terminus) and had a predicted size of 521 base pairs (bp). The *Sau*3AI fragment of this cDNA (392 bp) was inserted into the plasmid vector pUC19 (digested with *Bam*HI and *Hin*cII) and amplified in *Escherichia coli*. Digestion with *Eco*RI and *Hin*dIII gave a fragment (431 bp) that was purified on a 1.2% agarose gel and ^{32}P-labeled with an oligonucleotide labeling kit (Pharmacia).

Screening of cDNA Libraries. Transfected *E. coli* (XL1-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% $NaDodSO_4$, and 100 μg of denatured salmon sperm DNA per ml at 42°C for 4 hr. ^{32}P-labeled probe was added, and hybridization was continued overnight. Filters were

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Abbreviations: 12-HPETE, (12*S*)-12-hydroperoxy-(5*Z*,8*Z*,10*E*, 14*Z*)-5,8,10,14-eicosatetraenoic acid; HEL cells, 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).

washed twice at room temperature for 15 min with 2× SSC/0.1% $NaDodSO_4$ and then twice with 0.2× SSC/0.1% $NaDodSO_4$ 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 *Eco*RI 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-Lipoxygenase 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 1.5×10^5 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 $CaCl_2$/2 mM glutathione, pH 7.4, at a concentration of $5–20 \times 10^6$ 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 μM) 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/acetic acid, 350:150:3, vol/vol). After centrifugation, the supernatant was applied to a mini-ODS silica column (Chromabond C_{18}; 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/acetic 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-Lipoxygenase 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-^{32}P]dCTP by using random hexamers (oligolabeling 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 CDM8 (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-lipoxygenase cDNA was ligated to CDM8 (opened with *Bst*XI) by using a *Not* I/*Bst*XI adaptor prepared by annealing two oligonucleotides (5′-CTGGTACCGC and 5′-GGCCGCGGTACCAG-CACA). This gave the plasmid CDM8plT3, 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, Nord-Vacc, Skärholmen, Sweden) supplemented with 10% (vol/vol) fetal bovine serum. COS cells (8×10^5 cells) at 25% confluency in 100-mm dishes were transfected in 3.75 ml of OPTI-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-

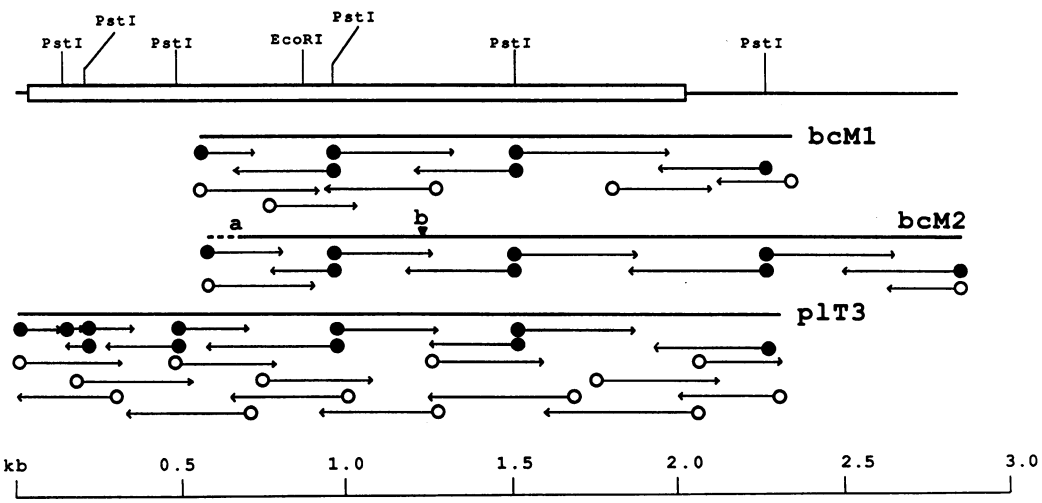

FIG. 1. Restriction map and sequencing strategy of human platelet 12-lipoxygenase cDNA. The protein coding region is indicated by an open bar. The closed circles indicate restriction sites, and the open circles indicate oligonucleotide primers. Direction and extent of sequence determination are indicated 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-lipoxygenase cDNA sequence (Fig. 2) was deduced from clones plT3 (nucleotides from −33 to 2289) and bcM1 (nucleotides 2290–2302).

line. 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 code (TGA), and a polyadenylylation code (AATAAA). 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′-. . . . .CTGGCTGAGAAG. . . . .-3′ of bcM1 was changed to 5′-. . . . .ACCAAAGAGAAG. . . . .-3′ in bcM2. Also, 4 bp (GCGA, 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 rela-

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                                                                    -33    CGGCTCCCCTCGCCTAAGCTGCTGGGGGGCGCC    -1

ATG GGC CGC TAC CGC ATC CGC GTG GCC ACC GGG GCC TGG CTC TTC TCC GGG TCG TAC AAC CGC GTG CAG CTT TGG CTG GTC GGG ACG CGC    90
Met Gly Arg Tyr Arg Ile Arg Val Ala Thr Gly Ala Trp Leu Phe Ser Gly Ser Tyr Asn Arg Val Gln Leu Trp Leu Val Gly Thr Arg    29

GGG GAG GCG GAG CTG GAG CTG CAG CTG CGG CCG GCG CGG GGC GAG GAG GAG GAG TTT GAT CAT GAC GTT GCA GAG GAC TTG GGG CTC CTG   180
Gly Glu Ala Glu Leu Glu Leu Gln Leu Arg Pro Ala Arg Gly Glu Glu Glu Glu Phe Asp His Asp Val Ala Glu Asp Leu Gly Leu Leu    59

CAG TTC GTG AGG CTG CGC AAG CAC CAC TGG CTG GTG GAC GAC GCG TGG TTC TGC GAC CGC ATC ACG GTG CAG GGC CCT GGA GCC TGC GCG   270
Gln Phe Val Arg Leu Arg Lys His His Trp Leu Val Asp Asp Ala Trp Phe Cys Asp Arg Ile Thr Val Gln Gly Pro Gly Ala Cys Ala    89

GAG GTG GCC TTC CCG TGC TAC CGC TGG GTG CAG GGC GAG GAC ATC CTG AGC CTG CCC GAG GGC ACC GCC CGC CTG CCA GGA GAC AAT GCT   360
Glu Val Ala Phe Pro Cys Tyr Arg Trp Val Gln Gly Glu Asp Ile Leu Ser Leu Pro Glu Gly Thr Ala Arg Leu Pro Gly Asp Asn Ala   119

TTG GAC ATG TTC CAG AAG CAT CGA GAG AAG GAA CTG AAA GAC AGA CAG CAG ATC TAC TGC TGG GCC ACC TGG AAG GAA GGG TTA CCC CTG   450
Leu Asp Met Phe Gln Lys His Arg Glu Lys Glu Leu Lys Asp Arg Gln Gln Ile Tyr Cys Trp Ala Thr Trp Lys Glu Gly Leu Pro Leu   149

ACC ATC GCT GCA GAC CGT AAG GAT GAT CTA CCT CCA AAT ATG AGA TTC CAT GAG GAG AAG AGG CTG GAC TTT GAA TGG ACA CTG AAG GCA   540
Thr Ile Ala Ala Asp Arg Lys Asp Asp Leu Pro Pro Asn Met Arg Phe His Glu Glu Lys Arg Leu Asp Phe Glu Trp Thr Leu Lys Ala   179

GGG GCT CTG GAG ATG GCC CTC AAA CGT GTT TAC ACC CTC CTG AGC TCC TGG AAC TGC CTA GAA GAC TTT GAT CAG ATC TTC TGG GGC CAG   630
Gly Ala Leu Glu Met Ala Leu Lys Arg Val Tyr Thr Leu Leu Ser Ser Trp Asn Cys Leu Glu Asp Phe Asp Gln Ile Phe Trp Gly Gln   209

AAG AGT GCC CTG GCT GAG AAG GTT CGC CAG TGC TGG CAG GAT GAT GAG TTG TTC AGC TAC CAG TTC CTC AAT GGT GCC AAC CCC ATG CTG   720
Lys Ser Ala Leu Ala Glu Lys Val Arg Gln Cys Trp Gln Asp Asp Glu Leu Phe Ser Tyr Gln Phe Leu Asn Gly Ala Asn Pro Met Leu   239
                                                  (G)                       (A)
TTG AGA CGC TCG ACC TCT CTG CCC TCC AGG CTA GTG CTG CCC TCA GGG ATG GAA GAG CTT CGG GCT CAA CTG GAG AAA GAA CTT CAG AAT   810
Leu Arg Arg Ser Thr Ser Leu Pro Ser Arg Leu Val Leu Pro Ser Gly Met Glu Glu Leu Arg Ala Gln Leu Glu Lys Glu Leu Gln Asn   269

GGT TCC CTG TTT GAA GCT GAC TTC ATC CTT CTG GAT GGA ATT CCA GCC AAC GTG ATC CGA GGA GAG AAG CAA TAC CTG GCT GCC CCC CTC   900
Gly Ser Leu Phe Glu Ala Asp Phe Ile Leu Leu Asp Gly Ile Pro Ala Asn Val Ile Arg Gly Glu Lys Gln Tyr Leu Ala Ala Pro Leu   299
                                                                                  (G)
GTT ATG CTG AAG ATG GAG CCC AAT GGG AAG CTG CAG CCC ATG GTC ATC CAG ATT CAG CCT CCC AAC CCC AGC TCT CCA ACC CCA ACA CTG   990
Val Met Leu Lys Met Glu Pro Asn Gly Lys Leu Gln Pro Met Val Ile Gln Ile Gln Pro Pro Asn Pro Ser Ser Pro Thr Pro Thr Leu   329

TTC CTG CCC TCA GAC CCC CCA CTT GCC TGG CTC CTG GCA AAG TCC TGG GTC CGA AAT TCA GAT TTC CAA CTG CAC GAG ATC CAG TAT CAC  1080
Phe Leu Pro Ser Asp Pro Pro Leu Ala Trp Leu Leu Ala Lys Ser Trp Val Arg Asn Ser Asp Phe Gln Leu His Glu Ile Gln Tyr His   359

TTG CTG AAC ACG CAC CTG GTG GCT GAG GTC ATC GCT GTC GCC ACC ATG CGG TGC CTC CCA GGA CTG CAC CCC ATC TTC AAG TTC CTG ATC  1170
Leu Leu Asn Thr His Leu Val Ala Glu Val Ile Ala Val Ala Thr Met Arg Cys Leu Pro Gly Leu His Pro Ile Phe Lys Phe Leu Ile   389
                                                  (A)
CCC CAT ATC CGC TAC ACC ATG GAA ATC AAC ACC CGG GCC CGG ACC CAA CTC ATC TCA GAT GGA GGA ATT TTT GAT AAG GCA GTG AGC ACA  1260
Pro His Ile Arg Tyr Thr Met Glu Ile Asn Thr Arg Ala Arg Thr Gln Leu Ile Ser Asp Gly Gly Ile Phe Asp Lys Ala Val Ser Thr   419

GGT GGA GGG GGC CAT GTA CAG TTG CTC CGT CGG GCG GCA GCT CAG CTG ACC TAC TGC TCC CTC TGT CCT CCT GAC GAC CTG GCT GAC CGG  1350
Gly Gly Gly Gly His Val Gln Leu Leu Arg Arg Ala Ala Ala Gln Leu Thr Tyr Cys Ser Leu Cys Pro Pro Asp Asp Leu Ala Asp Arg   449

GGC CTG CTG GGA CTC CCA GGT GCT CTC TAT GCC CAT GAT GCT TTA CGG CTC TGG GAG ATC ATT GCC AGG TAT GTG GAG GGG ATC GTC CAC  1440
Gly Leu Leu Gly Leu Pro Gly Ala Leu Tyr Ala His Asp Ala Leu Arg Leu Trp Glu Ile Ile Ala Arg Tyr Val Glu Gly Ile Val His   479

CTC TTC TAC CAA AGG GAT GAC ATA GTG AAG GGG GAC CCT GAG CTG CAG GCC TGG TGT CGG GAG ATC ACG GAG GTG GGG CTG TGC CAG GCC  1530
Leu Phe Tyr Gln Arg Asp Asp Ile Val Lys Gly Asp Pro Glu Leu Gln Ala Trp Cys Arg Glu Ile Thr Glu Val Gly Leu Cys Gln Ala   509

CAG GAC CGA GGT TTC CCT GTC TCC TTC CAG TCC CAG AGT CAA CTC TGC CAT TTC CTC ACC ATG TGC GTC TTC ACG TGC ACT GCC CAG CAT  1620
Gln Asp Arg Gly Phe Pro Val Ser Phe Gln Ser Gln Ser Gln Leu Cys His Phe Leu Thr Met Cys Val Phe Thr Cys Thr Ala Gln His   539

GCC GCC ATC AAC CAG GGC CAG CTG GAC TGG TAT GCC TGG GTC CCT AAT GCT CCA TGC ACA ATG CGG ATG CCC CCA CCC ACC ACC AAG GAA  1710
Ala Ala Ile Asn Gln Gly Gln Leu Asp Trp Tyr Ala Trp Val Pro Asn Ala Pro Cys Thr Met Arg Met Pro Pro Pro Thr Thr Lys Glu   569

GAT GTG ACG ATG GCC ACA GTG ATG GGG TCA CTA CCT GAT GTC CGG CAG GCC TGT CTT CAA ATG GCC ATC TCA TGG CAT CTG AGT CGC CGC  1800
Asp Val Thr Met Ala Thr Val Met Gly Ser Leu Pro Asp Val Arg Gln Ala Cys Leu Gln Met Ala Ile Ser Trp His Leu Ser Arg Arg   599

CAG CCA GAC ATG GTG CCT CTG GGG CAC CAC AAA GAA AAA TAT TTC TCA GGC CCC AAG CCC AAA GCT GTG CTA AAC CAA TTC CGA ACA GAT  1890
Gln Pro Asp Met Val Pro Leu Gly His His Lys Glu Lys Tyr Phe Ser Gly Pro Lys Pro Lys Ala Val Leu Asn Gln Phe Arg Thr Asp   629
      (A)
TTG GAA AAG CTG GAA AAG GAG ATT ACA GCC CGG AAT GAG CAA CTT GAC TGG CCC TAT GAA TAT CTG AAG CCC AGC TGC ATA GAG AAC AGT  1980
Leu Glu Lys Leu Glu Lys Glu Ile Thr Ala Arg Asn Glu Gln Leu Asp Trp Pro Tyr Glu Tyr Leu Lys Pro Ser Cys Ile Glu Asn Ser   659

GTC ACC ATC TGA GCCCTAGAGTGACTCTACCTGCAAGATTTCACATCAGCTTTAGGACTGACATTTCTATCTTGAATTTCATGCTTTCCTAAAGTCTCTGCTGCTAAGGCTCTAT  2095
Val Thr Ile                                                                                                              662

TTCCTCCCCCAGTTAAACCCCCTACATTAGTATCCCACTAGCCCAGGGGAGCAGTAAACTTTCTCTGCAAAGACTAGATCCTTTTTTACGCTTTGCAGACCGCATAGTCACTGTCTCAA  2214

CTACTCAGCTCTCCTGCTGCAGCATGAAGGCAGCCACAGACAACATGGAAATGAGTGTGACTATGTTCCAATAAAACTTTATGGACAC                                2302
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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 polyadenylylation signal AATAAA. Some heterogeneities were observed among the clones bcM1, bcM2, and plT3. Alternative DNA sequences are shown in parentheses (see also Table 1).

Table 1. Heterogeneity of cDNA for human 12-lipoxygenase

Position of heterogeneity	Codons and corresponding AA of clones		
	bcM1	bcM2	plT3
254	TCG	TCA	TCA
	Ser	Ser	Ser
260	CAG	CGG	CGG
	Gln	Arg	Arg
321	AGC	AAC	AAC
	Ser	Asn	Asn
403	CGG	CAG	CGG
	Arg	Gln	Arg
633	CTA	CTG	CTG
	Leu	Leu	Leu

AA, amino acid.

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 polyadenylylation signal in the gene for 12-lipoxygenase.

To obtain a full-length clone, a human platelet library was prepared. About 2×10^4 clones were screened by using the radiolabeled *Pst* I fragment (552 bp) of bcM1 as probe. Three positive clones were isolated; the clone plT3 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 plT3 (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 also because the upstream region of this ATG has similarity to the eukaryotic initiation site. Thus in plT3, 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 (polyadenylylation signal) was present at nucleotide 2282–2287. The open reading frame encoded a protein of 662 amino acids, excluding the first methionine residue, with a calculated M_r of 75,590. Minor heterogeneities in the cDNA sequences were observed between the clones bcM1, bcM2, and plT3 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-$(Xaa)_8$-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 15-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

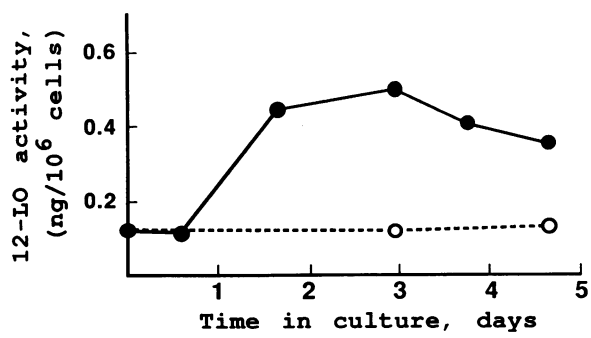

FIG. 3. Time course for induction of 12-lipoxygenase (12-LO) activity in HEL cells after differentiation. HEL cells were seeded at 1.5×10^5 cells per ml in the presence (●) or absence (○) of TPA at a concentration of 160 nM. The cells were harvested at the times indicated and assayed for 12-lipoxygenase activity as described.

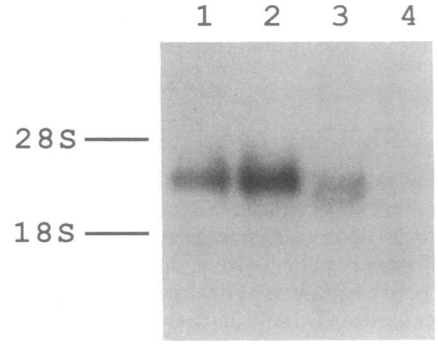

FIG. 4. RNA blot analysis of 12-lipoxygenase mRNA expression in HEL cells, platelets, and leukocytes. Poly(A)$^+$ RNAs (1.0 μg) from undifferentiated HEL cells (lane 1), differentiated HEL cells (80 nM TPA for 3 days) (lane 2), platelets (lane 3), and leukocytes (lane 4) were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to nitrocellulose, and processed as described. The *Not* I insert from clone plT3, 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.

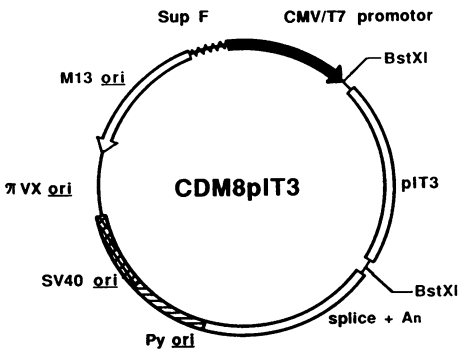

FIG. 5. Structure of the plasmid CDM8plT3 used for the expression of human platelet 12-lipoxygenase in COS cells. CMV/T7, cytomegalovirus/phage T7 RNA polymerase promoter; splice + An, splice and polyadenylylation signals from plasmid pSV2; Py ori, polyomavirus origin of replication; SV40 ori, simian virus 40 origin of replication; πVX ori, segment derived from pBR322 origin of replication; M13 ori, phage M13 origin of replication; SupF, *supF* gene.

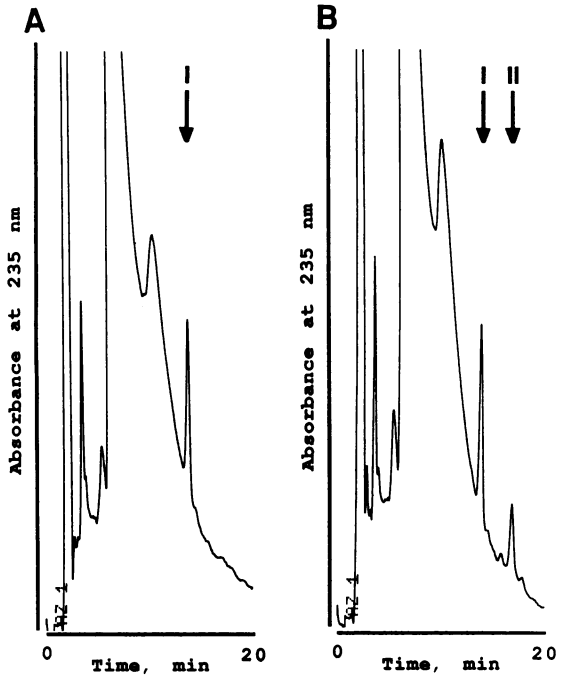

FIG. 6. HPLC analysis of 12-lipoxygenase activity of transfected COS cells. Two days after transfection with wild-type CDM8 (*A*) or CDM8plT3 (*B*), COS cells (4×10^5 cells) were incubated with arachidonic acid (160 μM). After precipitation of proteins, aliquots were analyzed by reversed-phase HPLC as described. Arrows indicate the positions of reduced activator (13-hydroxy-9,11-octadecadienoic acid) (I) and 12-HETE (II).

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 plT3 was introduced into the expression vector CDM8, giving CDM8plT3 (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 CDM8. 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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