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Murine epidermal lipoxygenase (*Aloxe*) encodes a 12-lipoxygenase isoform

Andreas Kinzig[a], Gerhard Fürstenberger[a], Friederike Bürger[a], Sonja Vogel[a], Karin Müller-Decker[a], Antoaneta Mincheva[b], Peter Lichter[b], Friedrich Marks[a], Peter Krieg[a,*]

[a] *Research Program on Tumor Cell Regulation, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*
[b] *Research Program on The Organisation of Complex Genomes, Deutsches Krebsforschungszentrum, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

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Abstract Using a combination of conventional screening procedures and polymerase chain reaction cloning, we have isolated a cDNA encoding an epidermis-type 12-lipoxygenase (*e12*-lipoxygenase) from mouse epidermis. The open reading frame corresponds to a protein of 662 amino acids and was found to be 99.8% identical to the ORF of an epidermal lipoxygenase gene *Aloxe*, described recently [Van Dijk et al. (1995) Biochim. Biophys. Acta 1259, 4–8]. When expressed in human embryonic kidney cells the recombinant protein could be shown to synthesize 12(*S*)-HETE from arachidonic acid. By fluorescence in situ hybridization the *e12*-lipoxygenase gene was localized to chromosome band 11 B1–B3.

Key words: Epidermis; Lipoxygenase; cDNA cloning; Functional expression; Chromosomal localization

1. Introduction

Lipoxygenases represent a family of non-heme iron-containing dioxygenases which stereospecifically insert molecular oxygen into *cis,cis*-1,4-pentadiene containing polyunsaturated fatty acids [1]. Mammalian lipoxygenases are classified according to the position of oxygen insertion into arachidonic acid yielding 5(*S*)-, 12(*S*)- and 15(*S*)-hydroperoxyeicosatetraenoic acids (HPETEs) as primary products. So far, 5- and 15-lipoxygenases and two isoforms of 12-lipoxygenase have been identified and cloned from several mammalian tissues [2–4]. The latter are different with respect to substrate and product specificity and were classified as platelet-type (*p12*-) and leukocyte-type (*l12*-)12-lipoxygenase.

Lipoxygenases were found to be predominantly expressed in hematopoietic cells. Recently, keratinocytes have been shown to be another abundant source of lipoxygenases. Expression of 5-lipoxygenase has been reported in human keratinocytes [5] and 8- and 15-lipoxygenase activities were found in tumor promoter-treated mouse epidermis [6] and in human and rat skin [7,8], respectively. Among the 12-lipoxygenases the platelet-type was found to be the predominant isozyme expressed in human and murine skin epidermis [9–11].

In this paper, we describe the cDNA cloning and functional expression of a novel lipoxygenase isoenzyme from mouse skin. The genomic sequence of this epidermal lipoxygenase (*Aloxe*) has been recently published by Van Dijk et al. [12]. Based on the enzymatic activities of the recombinant protein expressed in human embryonic kidney cells we conclude that this enzyme is a novel epidermis-type 12-lipoxygenase isoform (*e12*-lipoxygenase).

2. Materials and methods

2.1. cDNA clone isolation

Construction of cDNA library from pooled mouse skin papillomas and screening with a 1.23 kb 3′-*Eco*RI fragment of human platelet-type 12-lipoxygenase as ^{32}P-labeled probe was done as described earlier [11]. By screening of 8×10^5 recombinants (0.5×SSC, 0.5% SDS, 60°C, 20 min) one cDNA clone (Cl2014) was isolated which was different from previously isolated murine 12-lipoxygenase cDNA clones. The insert of 1.16 kb was sequenced and found to encode a lipoxygenase. The missing 5′-end was cloned by RT-PCR. Total RNA from mouse epidermis, which was shown to express abundantly Cl2014-related mRNA, served as template for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase and random primers. Polymerase chain reaction was carried out for 30 cycles in a Biometra thermal cycler with Taq polymerase at 91°C for 90 s, 54°C for 90 s, and 72°C for 150 s using a Cl2014-specific antisense primer (5′-AACACGACGTGGCTCTCTGGAG-3′) and a sense primer corresponding to the N-terminal sequence of porcine 12-lipoxygenase [13] (5′-ATGGGTCTCTACCGCGTCCG-3′) which exhibited the highest sequence similarity to Cl2014. The 1.39 kb DNA fragment amplified with these primers was subcloned into the pCRII-vector (Invitrogen) and sequenced. The PCR product surprisingly contained the Cl2014-specific antisense primer at both ends but revealed a continuous open reading frame and a sequence identity with Cl2014 in the 199 bp overlapping region indicating the correct product. A unique *Apo*I site in the overlapping region was utilized to combine the PCR generated clone with the cDNA clone to obtain a full length cDNA which was then subcloned into the mammalian expression vector pcDNA3 to give clone Cl2122. DNA sequencing was carried out by the dideoxy chain-termination method with phage T7 DNA polymerase (Pharmacia) to determine the entire sequence for both strands. Nucleotide and amino sequence analyses were carried out using the Heidelberg Unix Sequence Analysis Resources (HUSAR) software programs.

2.2. Fluorescence in situ hybridization (FISH)

A partial genomic clone of *e12*-lipoxygenase was isolated by screening a mouse genomic library (constructed in *EMBL3* with partial *Sau*3A digested NMRI mouse genomic DNA) with insert DNA of Cl2014 under high stringency conditions. A DNA fragment of 16 kb corresponding to the 5′-end of this genomic clone was analysed and found to contain exons 7–14 of the *e12*-lipoxygenase gene. For fluorescence in situ hybridization this DNA fragment was digoxigenin-labeled and hybridized to mouse metaphase chromosomes as described by Lichter et al. [14]. 80 ng of the labeled probe was combined with 3 μg of mouse Cot 1 DNA and 7 μg of salmon sperm DNA in a 10 μl hybridization cocktail. The hybridized probe was detected via rhodamine. Chromosomes were counterstained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). Images of the fluorescence signals and the DAPI counterstain were separately acquired by using a cooled CCD camera (Photometrics) and electronical overlaying of images were performed by using the software package: NIH image 1.41. Photographs were taken directly from the video screen.

*Corresponding author. Fax: (49) (6221) 42 4406.
E-mail: p.krieg@dkfz-heidelberg.de

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2.3. In vitro translation and immunoprecipitation

The plasmid DNA of Cl2122 was linearised with *Xho*I (sense orientation) and *Eco*RI (antisense orientation) and transcribed with T7 and T3 RNA polymerases, respectively, in the presence of the cap analogue. The sense and antisense transcripts were translated using a rabbit reticulocyte lysate (Stratagene) by the procedure recommended by the manufacturer in the presence of [^{35}S]Met. The primary translation products of the Cl2122 sense RNA was immunoprecipitated with a polyclonal rabbit antiserum against human platelet-type 12-lipoxygenase (Cayman Chemical) as described [15]. The primary translation products and the immunoprecipitate were analyzed on 7.5% SDS-polyacrylamide gels.

2.4. Western blot analysis

Proteins (50 μg) from cell homogenates were electrophoresed in 7.5% SDS-polyacrylamide gels. The proteins were electroblotted onto PVDF membranes and were subsequently treated as recommended by the supplier of the enhanced chemiluminescence detection system (Amersham Buchler). The anti-12-lipoxygenase antiserum (Cayman Chemical) was used at a dilution of 1:1000 and the anti-rabbit immunoglobulin G-alkaline peroxidase antibody (Jackson Immuno Research Lab.) at a dilution of 1:2000.

2.5. Expression of lipoxygenase cDNA

For transient expression plasmid DNA (10 μg) and vector DNA as control were introduced into human embryonic kidney (HEK) 293 cells (seeded at 10^6 per 100 mm plate) by a modified calcium phosphate transfection procedure using a mammalian transfection kit according to manufacturer's protocol (Stratagene). Cells were harvested 48 h after transfection and homogenized by sonication (Branson Sonifier, 6 sec on ice) in TE buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, pH 7.4).

2.6. Lipoxygenase assay conditions

Aliquots of 250 μl of cell homogenates were incubated in the presence of 20–40 μM [1-^{14}C]arachidonic acid or unlabeled fatty acid (100 μM) for 15 min at 37°C. The incubations were terminated by addition of 40 μl of 1 M sodium formate buffer (pH 3.1). To reduce the hydroperoxy acids to hydroxy acids 20 μl of trimethylphosphite were added and incubated at room temperature for 5 min. Samples were extracted twice with ethyl acetate. The combined organic extracts were evaporated to dryness, redissolved in 750 μl ethanol and diluted to a final ethanol concentration of 15% by addition of 0.1 M sodium formate buffer. An enriched HETE fraction was obtained by solid phase extraction according to Powell [16].

2.7. Analysis of HETEs

For high-performance liquid chromatography (HPLC) a Zorbax Sil column (250×4.6 mm, 5 μm, Bischoff) was eluted with 1 ml/min of a solvent system consisting of *n*-hexane/isopropanol/acetic acid/H_2O = 98.5:1.5:0.1:0.025 v/v). The eluant was monitored at 236 nm. For details see [17]. HETEs obtained by straight-phase analysis were analysed by chiral-phase chromatography using a Chiralcel column (250×4.6 mm, 5 μm, Baker) with the solvent system *n*-hexane/isopropanol/acetic acid = 99:1:0.05 (v/v). The eluant was monitored at 236 nm. Derivatization of the HETEs and GC/MS was performed as described previously [17].

3. Results and discussion

Screening of a lambda ZAP cDNA library derived from pooled mouse skin papillomas with a 1.23 kb 3′-*Eco*RI fragment of human platelet-type 12-lipoxygenase as probe re-

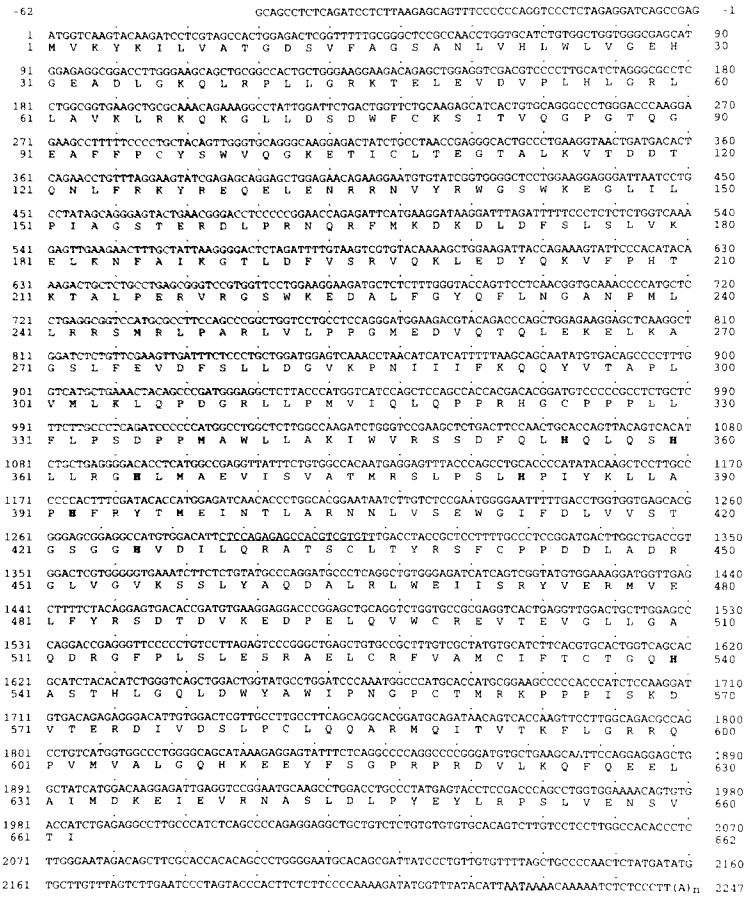

Fig. 1. Nucleotide sequence and deduced amino acid sequence of murine *e12*-lipoxygenase cDNA. Nucleotides and amino acids are numbered on the right beginning with the ATG initiation codon. Nucleotides on the 5′-side are designated by negative numbers. The deduced amino acid sequences are shown in the one-letter code below the nucleotide sequence. Conserved histidine residues found in all lipoxygenases are boldface. The position of the oligonucleotide primer used for the PCR amplification to complete the cDNA and the polyadenylation site are underlined.

vealed a cDNA clone (Cl2014), which was related to but different from previously isolated cDNA clones coding for the murine *p12*- and *l12*-lipoxygenase isoforms [11]. The insert was 1.16 kb in length. To obtain the missing 5′-end we performed RT-PCR with total RNA derived from mouse epidermis using a Cl2014-specific antisense primer and a sense primer based on the N-terminal sequence of porcine 12-lipoxygenase. The 1.39 kb DNA fragment amplified with these primers was subcloned into the pCRII vector (Invitrogen) and sequenced. A unique *Apo*I site in the overlapping region was utilized to combine the PCR generated clone with the cDNA clone to obtain a full length cDNA which was then subcloned into the mammalian expression vector pcDNA3. The combined cDNA (Fig. 1) consisted of a 5′-noncoding region (62 bp), a protein coding region (1986 bp including initiation ATG), and a 3′-untranslated region of 261 bp and was 2309 bp in size. The open reading frame encoded a protein of 662 amino acids, including the initiation methionine, with a calculated molecular mass of 75.45 kDa. As determined by progressive alignment of the predicted amino acid sequence the encoded protein was about equally related to 15-lipoxygenases and 12-lipoxygenases exhibiting 66% sequence identity to porcine [13] and 60% to murine *l12*-lipoxygenases [11,21], 66% identity to human and 62% identity to rabbit 15-lipoxygenase [18,19] as well as 60% identity to both, human and murine *p12*-lipoxygenases [11,21]. The lipoxygenase described here represents a distinct novel mammalian lipoxygenase isoform. Based on its in vivo expression pattern and the enzymatic activity of the recombinant protein (as shown below) we classified this enzyme as epidermis-type 12-lipoxygenase (*e12*-lipoxygenase). As shown for other cloned lipoxygenases the three His residues and the C-terminal Ile residue which have been proposed to be involved in iron binding [20] are found in *e12*-lipoxygenase at positions 360, 365, 540 and 662, respectively. Three other conserved His residues are at positions 355, 383, 392. Interestingly, we found a zinc-finger motif (C-X_3-C-X_3-H-X_3-H) at residues 532–544 which is also present in the 15-lipoxygenases [3] and in *l12*-lipoxygenases but is lacking in the *p12*-lipoxygenases [4]. Recently, Van Dijk et al. reported the complete sequence of a murine epidermal lipoxygenase gene, *Aloxe* [12]. The amino acid sequence of this lipoxygenase is identical to that of *e12*-lipoxygenase described in this paper except for one amino acid ($A^{619} \rightarrow P^{619}$). In addition, there are some differences in the 3′-untranslated region which might be due to polymorphism between the mouse strains used for the isolation of the *e12*-lipoxygenase cDNA (NMRI) and of the *Aloxe* gene (129Sv).

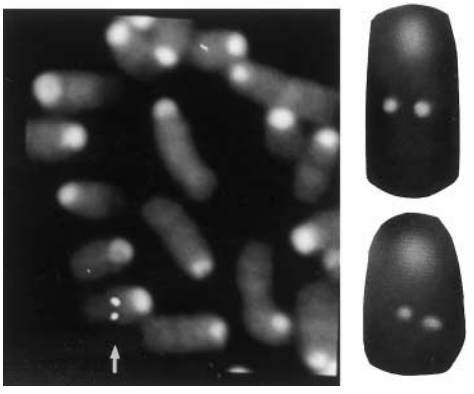

Fig. 2. Localization of murine *e12*-lipoxygenase to chromosome 11 B1–B3 by FISH. Chromosomes were counterstained with DAPI. In the left panel, a section of a mouse metaphase spread is shown, whereas on the right 2 chromosome 11 homologs are depicted to illustrate the band assignment.

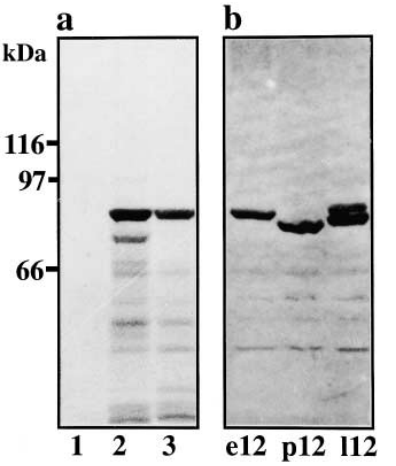

Fig. 3. Expression of recombinant epidermis-type 12-lipoxygenase. (a) Immunoprecipitation of in vitro translated epidermis-type 12-lipoxygenase: *e12*-lipoxygenase cDNA inserted into pCDNA3 was transcribed in both directions. Sense and antisense transcripts were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]Met. The translation products were immunoprecipitated as described in Section 2. The in vitro translation products (lane 1, antisense transcript; lane 2, sense transcript) and the precipitated proteins of the sense transcript (lane 3) were separated on a 7.5% SDS-polyacrylamide gel, and the gel was dried and autoradiographed. (b) Immunoblot of murine 12-lipoxygenase isoenzymes expressed in HEK 293 cells. Cell homogenate protein (50 μg) from sonicated HEK 293 cells transfected with cDNA expression constructs of murine epidermis-type (e12), platelet-type (p12) and leukocyte-type (l12) 12-lipoxygenase were separated on a 7.5% SDS-polyacrylamide gel, blotted onto PVDF membrane and incubated with a polyclonal anti-human 12-lipoxygenase antiserum.

The chromosomal localization of the *e12*-lipoxygenase gene was determined by fluorescence in situ hybridization as described [14] using a digoxigenin-labeled genomic DNA fragment of *e12*-lipoxygenase. In 85% of the analyzed metaphase cells fluorescent signals were detected on one of the homologues of chromosome band 11 B1–B3 whereas in 15% of the cells signals were found on both homologues of chromosome 11. No other signals could be detected in other areas of the mouse genome (Fig. 2). Recently, the murine genes for platelet-type (*Alox12p*) and leukocyte-type 12-lipoxygenase (*Alox12l*) have been mapped to the central region of chromosome 11 by Chen et al. [21]. A third gene in this locus (*Alox12-ps1*) was thought to be non-functional due to the apparent absence of exon 12.

The full-length cDNA of *e12*-lipoxygenase under the control of the immediate-early cytomegalovirus promoter was transiently transfected into HEK 293 cells. Expression of the recombinant protein can be detected as a ≈77 kDa protein by an anti-human *p12*-lipoxygenase antiserum which was shown to immunoprecipitate the in vitro translated *e12*-lipoxygenase (Fig. 3a). Using this antiserum the recombinant murine *p12*-lipoxygenase was detected as a ≈75k Da band and the murine *l12*-lipoxygenase as a doublet of ≈76 and 78 kDa. A similar expression level of the three recombinant proteins is indicated by comparison of the staining intensities (Fig. 3b).

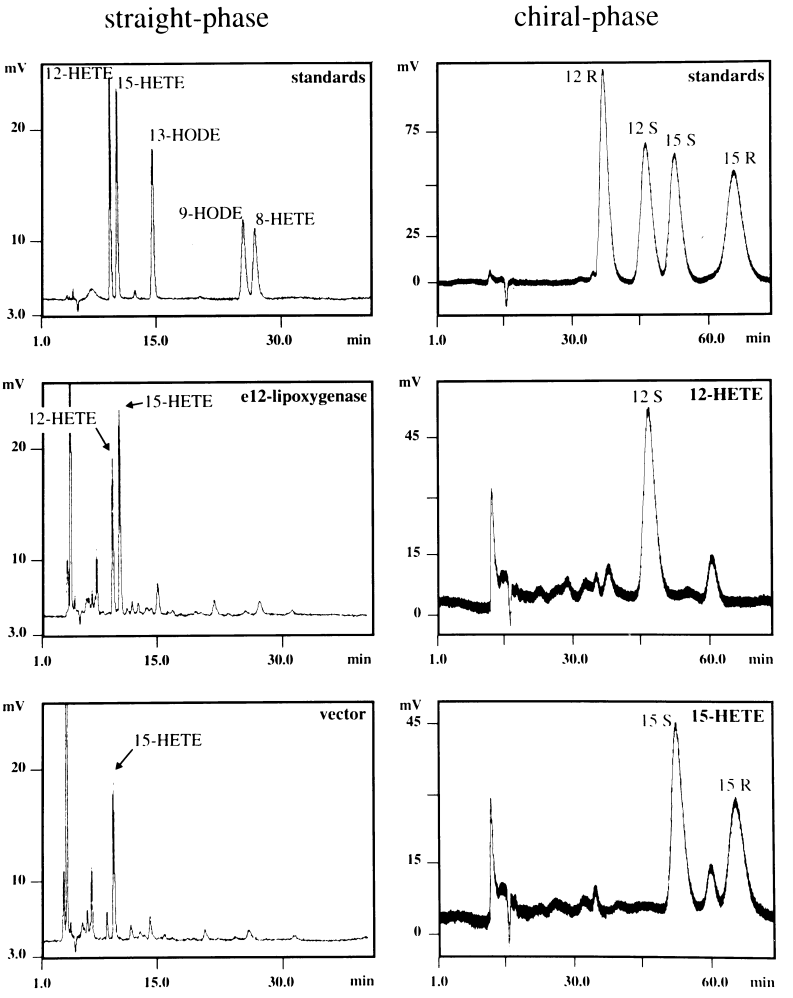

Fig. 4. Straight- and chiral-phase HPLC chromatograms of the products formed from murine *e12*-lipoxygenases expressed in HEK 293 cells. Homogenates from HEK 293 cells transiently transfected with constructs containing *e12*-lipoxygenase cDNA or with vector constructs without insert were incubated in TE buffer with 100 μM arachidonic acid for 15 min at 37°C. H(P)ETEs were reduced and extracted according to the method of Powell. The HETE fraction was analysed on a Zorbax Sil straight-phase HPLC column as described in Section 2. 8-, 12- and 15-HETE and 9- and 13-HODE were used as internal standards. The retention times of 12-HETE and 15-HETE were 9.19 and 10.02 min, respectively. The identity of 12-HETE was confirmed by GC/MS chromatography. The 12-HETE and 15-HETE eluates were subjected to chiral-phase chromatography on a Chiracel column using 12*S*-, 12*R*-, 15*S*- and 15*R*-HETE as internal standards. The retention times of 12*R*- and 12*S*-HETE were 37.26 and 46.42 min, respectively, those of 15*S*-HETE and 15*R*-HETE being 52.77 and 65.47 min.

Homogenates of HEK cells obtained 48 h after transfection with the *e12*-lipoxygenase construct were incubated with arachidonic acid and the reduced products were analysed by straight-phase and chiral-phase HPLC and by gas chromatography/mass spectrometry. Incubation with arachidonic acid resulted in the formation of 12-HETE and 15-HETE. Cells transfected with the negative control plasmid (lacking a cDNA insert), did not yield any 12-HETE but the same amount of 15-HETE. Chiral phase analyses showed that the *S*-enantiomer was the almost exclusive 12-HETE product while 15-HETE was a racemic mixture (Fig. 4) indicating that 15-HETE was not a product of a lipoxygenase activity. With a substrate conversion rate of 8–12% the catalytic activity of the *e12*-lipoxygenase was about one-fifth of that of the murine *p12*-isoenzyme (50–65% substrate conversion). The conversion rate of linoleic acid was below 2% for both isoenzymes. The low catalytic activity of *e12*-lipoxygenase as compared to *p12*-lipoxygenase may indicate that keratinocyte-specific cofactors or post-translational modifications are needed to obtain optimal activity. Alternatively, yet unknown polyunsaturated fatty acids or complex lipids may be the preferential substrates for this enzyme.

By Northern blot analysis a 2.4 kb transcript corresponding to *e12*-lipoxygenase was detected in epidermis but not in samples from other epithelia like intestine, liver, kidney or lung or in samples from spleen, adipose, muscle and brain. We also failed to detect any expression of *e12*-lipoxygenase-specific transcripts in murine lymphocytes and reticulocytes (not shown). These data are in agreement with the expression pattern of *Aloxe* reported by Van Dijk et al. [12].

In summary, we have cloned the cDNA encoding a murine lipoxygenase which is exclusively expressed in epidermis. Based on sequence homology data the protein is about equally related to the leukocyte-type and the platelet-type murine 12-lipoxygenases (60% sequence identity). Upon transfection into HEK cells the enzyme exhibits a 12-lipoxygenase activity. Thus, this epidermal lipoxygenase represents a third isoform of mammalian 12-lipoxygenases. With respect to substrate and product specificity the *e12*-lipoxygenase is more related to the platelet-type than to the leukocyte-type enzyme.

4. Note added in proof

While this paper was in preparation, Funk et al. described the cDNA cloning and expression of a mouse epidermal lipoxygenase [22]. Our data confirm the assumption of the authors that murine epidermal lipoxygenase is a 12-lipoxygenase.

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