Prostaglandins, Leukotrienes and Essential Fatty Acids 138 (2018) 14–23

Contents lists available at ScienceDirect

Prostaglandins, Leukotrienes and Essential Fatty Acids

journal homepage: www.elsevier.com/locate/plefa

Do lipoxygenases occur in viruses? Expression and characterization of a viral lipoxygenase-like protein did not provide evidence for the existence of functional viral lipoxygenases

Tatjana Gehring[a], Dagmar Heydeck[a], Agathe Niewienda[b], Katharina Janek[b], Hartmut Kuhn[a,*]

[a] Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Institute of Biochemistry, Chariteplatz 1, D-10117 Berlin, Germany

[b] Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Core facility for Mass Spectrometry, Chariteplatz 1, D-10117 Berlin, Germany

ARTICLE INFO

Keywords:
Eicosanoids
Oxidative stress
Horizontal gene transfer
Genomes development
Radicals

ABSTRACT

Lipoxygenases are lipid peroxidizing enzymes, which frequently occur in higher plants and animals. In bacteria, these enzymes are rare and have been introduced *via* horizontal gene transfer. Since viruses function as horizontal gene transfer vectors and since lipoxygenases may be helpful for releasing assembled virus particles from host cells we explored whether these enzymes may actually occur in viruses. For this purpose we developed a four-step *in silico* screening strategy and searching the publically available viral genomes for lipoxygenase-like sequences we detected a single functional gene in the genome of a mimivirus infecting *Acantamoeba polyphaga*. The primary structure of this protein involved two putative metal ligand clusters but the recombinant enzyme did neither contain iron nor manganese. Most importantly, it did not exhibit lipoxygenase activity. These data suggests that this viral lipoxygenase-like sequence does not encode a functional lipoxygenase and that these enzymes do not occur in viruses.

1. Introduction

Lipoxygenases (ALOX-isoforms) are fatty acid dioxygenases, which oxygenate polyunsaturated fatty acids to corresponding hydroperoxy derivatives [1–3]. They occur in two domains of life on earth (bacteria, eukarya) but have not been detected in archaea [4]. ALOX-isoforms are expressed at high frequency in mammals and higher plants but they rarely occur in lower organisms [4]. In mammals, ALOX-isoforms frequently occur [4] and mammalian ALOXs have been implicated in cell maturation [5,6] and differentiation [7–10]. They also play a role in the pathogenesis of inflammatory [11–13], hyperproliferative [14–16] and neurological diseases [17–20]. The human genome [21] involves 6 functional ALOX genes (ALOX15, ALOX15B, ALOX12, ALOX12B, ALOX5, ALOXE3). Except for ALOX5, which is localized on chromosome 10, all human ALOX genes have been mapped to a joint ALOX cluster located on chromosome 17. In mice there is a corresponding ortholog for each human ALOX gene [21]. Interestingly, the Alox12e gene, which is corrupted in humans, constitutes a functional gene in mice and rats.

In lower plants ALOX-like sequences are also rare but higher plants frequently express lipoxygenase-isoforms [4]. The most comprehensively characterized plant lipoxygenase is soybean LOX1 [22,23]. Completion of the cucumber genome identified more than 20 lipoxygenase genes [24]. Similarly, the genomes of *Glycine* max and *Medicago truncatula* involve more than 30 lipoxygenase genes, which have been mapped to different gene clusters [25]. Classification of plant lipoxygenases is rather difficult, but based on sequence comparison two major types of plant lipoxygenases have been classified [25,26]. Type-1 lipoxygenases share a high degree of amino acid sequence similarity (> 75%) and do not carry a plastidic transit peptide. In contrast, type-2 lipoxygenases have such chloroplast transit peptide but only share a low degree of amino acid conservation (< 35%). The biological function of plant lipoxygenases has also been studied in detail. The enzymes have been implicated in the biosynthesis of oxylipins, which are involved in stress response [27], growth and development [28], germination [29], tuber formation [30], sex determination [31], senescence [32], fruit ripening [33] and aroma production [34].

A systematic search for lipoxygnease-like sequences in bacterial genomes suggested that less than 0.5% of all bacterial genomes involve functional lipoxygenase genes. Although functional lipoxygenases have

* Corresponding author.
E-mail address: hartmut.kuehn@charite.de (H. Kuhn).

https://doi.org/10.1016/j.plefa.2018.10.002
Received 4 April 2018; Received in revised form 24 July 2018; Accepted 3 October 2018

been characterized in several bacterial species [35–38] their biological functions largely remain elusive [39]. Most human pathogenic bacteria do not carry functional lipoxygenase genes [4] and thus these enzymes may not be relevant as molecular drug targets for frequently occurring infectious diseases. An exception from this rule is *Pseudomonas aeruginosa*. This facultative pathogen, which frequently infects immunocompromized patients, involves a functional lipoxygenase gene encoding for a secreted enzyme [35]. This enzyme has been characterized comprehensively and its crystal structure indicated a bifurcated catalytic center, in which an endogenous phospholipid ligand is bound [38,39]. The enzyme exhibits a low oxygen affinity [40], which is characteristic for sensor proteins, and is capable of decomposing human erythrocytes, when incubated *in vitro* with these cells [41].

Unfortunately, there is hardly any information currently available as to the occurrence of lipoxygenases in viruses. This lack of knowledge is somewhat surprising because of several reasons: (i) Viruses are the most frequently occurring biological systems on the planet [42] and in most biotopes the number of virus particles is typically 10–100 fold higher than the number of living cells [43–45]. (ii) Viruses are important vehicles for horizontal gene transfer [46,47] and lipoxygenases have probably been introduced into bacteria *via* horizontal gene transfer [47,48]. (iii) Given the abundant occurrence of lipoxygenase genes in higher plants and mammals there is a high probability that viruses should have captured corresponding sequences during evolution. (iv) Some lipoxygenase isoforms [29,41,49] are capable of oxidizing complex ester lipids and destabilize the structure of biomembranes. A virus infection involves the liberation of newly assembled virus particles and expression of a membrane attacking lipoxygenase might be helpful to initiate this process. To explore whether lipoxygenase genes occur in viruses we worked out a 4-step *in silico* screening strategy to identify functional lipoxygenase sequences in viral genomes. Employing this strategy we identified a potential viral lipoxygenase-like sequence in the genome of a mimivirus infecting *Acantamoeba polyphaga*, which is also present in other viruses (Samba virus, Niemeyer virus, Hirudovirus). However, when we expressed the corresponding protein in *E. coli*, we neither detected iron nor manganese in the final enzyme preparation and lipoxygenase-activity assays were negative. Thus, our results suggest that functional lipoxygenases may not occur in viruses.

2. Materials and methods

Chemicals - The chemicals used were obtained from the following sources: Antibiotics (chloramphenicol, ampicillin, kanamycin) were purchased from Carl Roth GmbH (Karlsruhe, Germany), Life Technologies, Inc. (Eggenstein, Germany) and Sigma Aldrich (Hamburg, Germany), respectively. Isopropyl-β-thiogalactopyranoside (IPTG) was obtained from Carl Roth GmbH (Karlsruhe, Germany) and sodium borohydride from Sigma–Aldrich (Hamburg, Germany), HPLC solvents were ordered from Fisher Scientific GmbH (Schwerte, Germany). Restriction enzymes were purchased from Thermo Scienific (Schwerte, Germany). Oligonucleotide synthesis was performed at BioTez (Berlin, Germany) and DNA sequencing was carried out at MWG Eurofins (Ebersberg, Germany). The cDNA encoding for the putative mimivirus lipoxygenase was chemically synthesized by Biomatik Corp. (Cambridge, Ontario, Canada). It was provided as lyophilized powder in the pBME cloning vector.

In silico screening strategy – To search for functional viral lipoxygenase genes we initially screened the NCBI viral genome databases (https://www.ncbi.nlm.nih.gov/pmc/) for lipoxygenase-like sequences using the search item “lipoxygenase” (1. step). This verbal search was based on well-defined classification algorithms of the database involving complex sequence comparisons with previously identified lipoxygenase sequences. The hits of this sequence-based screening were then taken through a three-level filtering strategy to remove false positive results: (i) 1st filtering step: We excluded all sequences, which encode for proteins consisting of < 300 amino acids. Eukaryotic lipoxygenases usually consist of 600–850 amino acids and the identified bacterial lipoxygenases are in a similar molecular weight range [35,36]. The miniLOX of Nostoc PCC7120 is somewhat smaller but still falls into this molecular weight range [37]. This filtering step (step 2 of our search strategy) eliminated incomplete lipoxygenase sequences, which most likely do not encode for functional lipoxygenases. (ii) 2nd filtering step: The sequences surviving the first filtering step were retrieved and inspected for the presence of metal coordinating amino acid clusters. Most lipoxygenases characterized so far carry two metal (iron or manganese) ligand clusters [cluster 1 (Clu1), **His**-AS1-AS2-AS3-AS4-**His**; cluster 2 (Clu2), **His**-ASa-ASb-ASc-**Asn** (His, Ser, Thr)] and each cluster involves two metal coordinating amino acids (bold face). The metal binding clusters of Fe- and Mn-containing lipoxygenases are very similar [50]. We eliminated all sequences from further consideration, which did not involve two metal binding clusters. (iii) 3rd filtering step: The sequences that survived the first two filtering steps were taken through a final round of selection, which was the distance of the two metal binding clusters. For most eukaryotic lipoxygenases the distance between the two metal binding clusters varied between 180 and 200 amino acids regardless whether they contain iron or manganese as catalytic active metal. Thus, we eliminated all sequences, in which the distance of the two metal binding clusters was smaller than 150 or larger than 250 amino acids. When we applied this 4-step screening strategy to the publically available viral genomes we filtered out a single sequence, which was present in the genome of different viruses. We named this sequence putative mimivirus lipoxygenase.

Subcloning strategy and construction of the expression plasmid – The coding region of the cDNA sequence of the putative mimivirus lipoxygenase was first optimized *in silico* for prokaryotic expression and an internal Hind III restriction site was eliminated by silent mutation. The resulting sequence was chemically synthesized and inserted into the pBME cloning vector. For bacterial expression the coding region was cut off and subcloned into the multicloning site of the pET28b bacterial expression vector between the restriction sites of Sal I (N-terminus) and Hind III (C-terminus). The expression vector contains both a 5′ (N-terminal) and a 3′- (C-terminal) hexa-his-tag sequence. We cloned the coding sequence of the potential mimivirus lipoxygenase into the expression vector in such a way that the coding sequence was in frame with the 5′-hexa-his-tag sequence. After subcloning the 3′-hexa-his-tag sequence was located behind the stop codon of the coding sequence of the putative mimivirus lipoxygenase and thus, this sequence was not transcribed. This strategy is absolutely essential for expression of functional recombinant lipoxygenases since for all lipoxygenases crystallized so far the C-terminal amino acid functions as iron ligand and thus the 3′-region of the coding region of the cDNA should not be modified. The final amino acid sequence of the lipoxygenase insert is given in Fig. 1. Because of technical reasons related to the subcloning strategy additional amino acids were introduced at the N-terminus of the recombinant protein so that the final amino acid sequence of the recombinant putative mimivirus lipoxygenase reads: Met-Gly-Ser-Ser-*His-His-His-His-His-His*-Ser-Ser-Gly-Leu-Val-Pro-Arg-Gly-Ser-His-Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Asp-Pro-Asn-Ser-Ser-Ser-Val-Asp-**Met-Leu-Lys-Lys.** The residues marked in bold represent the N-terminal amino acids of the original sequence of the mimivirus protein.

Recombinant expression – The recombinant expression plasmid was amplified and competent *E. coli* cells (BL21DE3pLysS, Rosetta2DE3pLysS) were transformed. After overnight incubation on kanamycine and chloramphenicol containing agar plates, well-separated bacterial clones were picked and the bacteria were grown in two 1 ml precultures conatining antibiotics. After 6 h of pre-culture 2 ml were taken and 50 ml main cultures were grown overnight. For expression of the recombinant protein the EnPresso B kit (BioSilta Ltd., St. Ives, Great Britain) was employed following the instructions of the vendor. To induce expression 1 mM IPTG was added to the main