Peptides 83 (2016) 21–28

Contents lists available at ScienceDirect

Peptides

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

Overexpression of Leap2 impairs *Xenopus* embryonic development and modulates FGF and activin signals

Pierre Thiébaud[a,b,1], Bertrand Garbay[c,1], Patrick Auguste[a,b], Caroline Le Sénéchal[c], Zuzanna Maciejewska[a,b], Sandrine Fédou[a,b], Xavier Gauthereau[a,d], Patricia Costaglioli[c,2], Nadine Thézé[a,b,*,2]

[a] *Univ. Bordeaux, F-33076 Bordeaux, France*
[b] *INSERM U1035, F-33076 Bordeaux, France*
[c] *Bordeaux INP, BPRVS, EA4135, F-33000 Bordeaux, France*
[d] *CNRS UMS 3427, F-33076 Bordeaux, France*

ARTICLE INFO

Article history:
Received 27 March 2016
Received in revised form 15 June 2016
Accepted 17 June 2016
Available online 19 June 2016

Keywords:
Leap2
Xenopus laevis
Embryo
FGF
Activin
Cell migration
HUVEC

ABSTRACT

Besides its widely described function in the innate immune response, no other clear physiological function has been attributed so far to the Liver-Expressed-Antimicrobial-Peptide 2 (LEAP2). We used the *Xenopus* embryo model to investigate potentially new functions for this peptide. We identified the amphibian *leap2* gene which is highly related to its mammalian orthologues at both structural and sequence levels. The gene is expressed in the embryo mostly in the endoderm-derived tissues. Accordingly it is induced in pluripotent animal cap cells by FGF, activin or a combination of vegT/β-catenin. Modulating leap2 expression level by gain-of-function strategy impaired normal embryonic development. When overexpressed in pluripotent embryonic cells derived from blastula animal cap explant, leap2 stimulated FGF while it reduced the activin response. Finally, we demonstrate that LEAP2 blocks FGF-induced migration of HUman Vascular Endothelial Cells (HUVEC). Altogether these findings suggest a model in which LEAP2 could act at the extracellular level as a modulator of FGF and activin signals, thus opening new avenues to explore it in relation with cellular processes such as cell differentiation and migration.

1. Introduction

Liver-expressed antimicrobial peptide 2 (LEAP2) was originally identified as a circulating peptide in human blood [1]. It is predominantly expressed in the liver as a 77-amino-acid pre-propeptide and is then processed into a 55-amino-acid propeptide, which is finally converted into the mature LEAP2 peptide of 40 residues. The mature human peptide is cationic (isoelectric point = 9.2) with four positive charges at pH 7.0 and contains two intramolecular disulfide bonds that are cross-linked in a 1–3, 2–4 pattern. Determination of the 3D structure by NMR spectroscopy identified a new fold with a central core stabilized by the two disulfide bonds and a network of hydrogen bonds [2].

The biological role of LEAP2 is not completely understood. A first report demonstrated its antimicrobial activity against some bacteria and fungi [1]. However, it was later demonstrated that the antibacterial activity of human LEAP2 is moderate, as the Minimum Inhibitory Concentration (MIC) measured *in vitro* are in the 50–100 μM range [2,3]. Moreover, it has been found that the sequence of the mature LEAP2 peptide is highly conserved among mammals and birds, and that orthologues can be found in reptiles and several species of fish [1,4–12]. This high level of conservation is quite unusual for antimicrobial peptides because they usually evolve in each animal species in order to adapt to its specific pathogens. Indeed, LEAP2 induction differs in response to bacterial infection depending on the species considered [7]. In this respect, it closely resembles another human peptide, LEAP1/hepcidin, a small peptide present in human blood that was initially identified as an antimicrobial peptide but which regulates iron homeostasis in mammals [13]. By analogy, it has been proposed that LEAP2 may have an additional biological function to its antibacterial activity. However, it has been recently reported that human LEAP2 does not play any role in cell proliferation and does not exhibit any chemo-

* Corresponding author. Univ. Bordeaux, INSERM U1035, F-33076 Bordeaux, France.
E-mail address: Nadine.theze-thiebaud@u-bordeaux.fr (N. Thézé).
[1] These authors contributed equally to this work.
[2] These authors share senior authorship.

http://dx.doi.org/10.1016/j.peptides.2016.06.008
0196-9781/

tactic activity on monocytes [14]. Finally, a role for LEAP2 during embryonic development cannot be ruled out because the corresponding transcripts were detected in chicken embryos, and at high level in early embryonic stages of the blunt snout bream [6,11].

LEAP2 mutants could be helpful to obtain insights into protein function but no natural mutation has yet been reported and no knock-out mouse for *Leap2* is currently available. To circumvent this problem, we decided to turn to a simple vertebrate model that allows efficient gain-of-function and loss-of-function strategies. We investigated leap2 in the amphibian *Xenopus laevis*. *Xenopus* has emerged as one of the most tractable models for the study of vertebrate embryogenesis thanks to its numerous advantages such as rapid development and the possibility to overexpress gene products through mRNA injection and a morpholino antisense oligonucleotide-mediated gene knockdown assay [15–17]. Moreover, animal cap explant from blastula embryo is a useful model of pluripotent cells that can be used to investigate signaling pathways that function in normal development [18]. In the present work, we report the structure and expression of the *leap2* gene in *Xenopus* and provide experimental data about its potential function in vertebrate development. Embryos in which leap2 has been overexpressed display developmental defects. We found that in pluripotent animal cap cells, leap2 stimulates the FGF signal while it inhibits activin signaling. Finally, we provide data that sustain a role of human LEAP2 in the down-regulation of FGF2 induced cell migration. Together our data constitute the first description of new functions for LEAP2 in mammals in cell signaling regulation and unveils unexpected physiological role for this peptide.

2. Material and methods

2.1. Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the European Community. The protocol was approved by the "Comiteí d'éthique en expérimentation de Bordeaux" N°33011005-A.

2.2. Embryonic manipulations

Xenopus laevis oocytes and embryos were obtained and staged using standard procedures and cultured in OR2 buffer and 0.1XMMR respectively [19,20]. Stage 36 embryos were dissected on agarose-coated Petri dish with forceps and pieces were directly lysed in TNES buffer before RNA extraction [20]. Stage 45/46 *Xenopus* embryos guts were dissected out and washed in 0.1XMMR before fixation for 1 h in MEMFA solution and stored in 100% ethanol [21].

2.3. Cloning and sequence analysis

Total RNA was isolated from *X. laevis* intestine as previously described [22]. 1 μg of RNA was reverse-transcribed with the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies) using a mix of oligo dT and random nonamer primers at a 5:1 ratio (w/w). One μl aliquot from the 20 μl single-stranded cDNA reaction and the corresponding RT negative control was diluted into 49 μl of PCR reaction mix. 30 amplification cycles (30 s at 95 °C, 45 s at 55 °C, and 90 s at 72 °C) were performed using a iCycler (Biorad) and Taq DNA polymerase (New England Biolabs). Primers used for *leap2* amplification were based on the EST sequence of leap2 from *Xenopus laevis* (GenBank: DT081114.1): F 5′- GGGATCC**ATG**TTCCTGCAGCCTGG-3′ and R 5′- GGAATTCTTACCTGCACAACTTGG-3′. The underlined sequences represent sites for the restriction enzymes BamHI and EcoRI. The bold sequence represents the initiation codon. These two primers are located respectively in exon 1 and exon 2 of the *Xenopus leap2* gene. After amplification, the PCR product was purified with Nucleospin Gel and PCR clean-up (Macherey-Nagel), ligated into pGEM-T vector (Promega) using Quick Ligase (New England Biolabs), and the plasmid was then electroporated into JM109 *E. coli* competent cells (Promega). Clones were thereafter selected and sequenced (MilleGen Biotechnologies, Toulouse, France). RACE experiments were performed using the Gene RacerTM kit (Invitrogen) and *Xenopus laevis* intestine RNA (2.5 μg/experiment). PCR products were cloned by using the TOPO TA Cloning Kit (Invitrogen) before sequencing. All experiments were performed according to the manufacturer's instructions. The *leap2* specific primers used for the RACE experiments were: 5′-AGGGGCCTGTCTTTGCGCCCATTAG-3′ (3′RACE) and 5′-GCGCAAAGACAGGCCCCTCCAAAAT-3′ (5′ RACE).

Xenopus leap2 sequences were retrieved from GenBank with the alignment search tool tBlastN from the National Center for Biotechnology Information (NCBI). Synteny analysis was performed using Ensembl and metazome and the gene structure was deduced by comparison between genomic and cDNA sequences.

2.4. Constructs, mRNA and injections

Xenopus leap2 cDNA was subcloned into BamHI and XbaI sites of pCS2 plasmid (pCS2-*leap2*) (F1 5′-GCGGGATCCATGTTCCTGCAGCCTGGG-3′; R1 5′-GCGTCTAGAATTACTAAAAGTCTTCAGCGACCAG-3′). For microinjection experiments, capped mRNAs were synthesized using the mMessenger mMachine SP6 Kit (Ambion) from Acc65I-linearized plasmids. For the overexpression study, 4-cell stage embryos were injected with 2.5 ng of leap2 mRNA. To identify the injected side, n*lacZ* (250 pg) were used as tracer and *lacZ* expression was revealed with the Red-Gal substrate (Research Organics).

For the animal caps assay, 2.5 ng of *leap2* mRNA, 500 pg of *vegT*, 200 pg of β-*catenin* or 1 ng of *XFD* mRNAs were injected into the animal pole of 2-cell stage embryos. In control experiments, *lacZ* mRNA was injected at the same dose than *leap2* or *vegT* + β-*catenin* mRNAs. Animal cap explants were dissected from stage 8–9 embryos and cultured until the control embryos reached the appropriate stage before RT-qPCR analysis. For growth factor treatment, FGF2 (100 ng/ml final) and activin (50 or 100 ng/ml final) were provided by R&D Systems. For inhibitor treatment, SU5402 (Calbiochem) was dissolved in DMSO (50 mM stock solution) and diluted in 0.1XMBS to 50 μM for animal cap treatment. U0126 and PD98059 were dissolved at 20 mM and 100 mM respectively before dilution to 50 μM.

2.5. RT-PCR, in situ hybridization

Total RNA was isolated from *Xenopus laevis* embryo and adult tissues as previously described [22]. RNA concentration was determined by spectrophotometry and integrity was assessed by agarose gel electrophoresis. For each sample, 1 μg of total RNA was reverse-transcribed with the AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies) using a mix of oligo dT and random nonamer primers at a 5:1 ratio (w/w). To evaluate a possible genomic DNA contamination, an RT negative control was generated for each RNA sample and used as a control in the PCR experiments. PCR was performed as described above with the primer pairs listed in Table S1. 5 μl of PCR reactions was analyzed on a 2% agarose gel containing ethidium bromide in TBE buffer and documented with a Gel Documentation System (Biorad). *In situ* hybridization was carried out as previously reported [23]. Antisense and sense probes were made from pCS2-*leap2* linearized plasmid with BamHI (+T7 RNA polymerase) and XbaI (+SP6 RNA polymerase) respec-