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Characterization of rat LANCL1, a novel member of the lanthionine synthetase C-like protein family, highly expressed in testis and brain

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Abstract

We isolated and characterized the cDNA coding for rat LANCL1, a new member of the eukaryotic LanC-like protein family which is related to the bacterial lanthionine synthetase components C (LanC). LanC is involved in the synthesis of antimicrobial peptides. Rat LANCL1 showed 91.5% and 96% identity when compared with the previously characterized human and mouse orthologs, respectively. Northern blot analysis revealed the presence of two major transcripts, at 1.5 kb and 5 kb, probably arising from the usage of two different polyadenylation signals. The 1.5 kb mRNA is massively expressed in testis, whereas the 5 kb transcript is most abundant in brain. The high level of expression of rat LANCL1 in these tissues was confirmed by Western blotting. *In situ* hybridization analyses of various rat tissues revealed a strong signal in the germinal cells of the seminiferous tubules in testis, in the neurons of the cerebellum, in liver hepatocytes, and in cardiac myocytes. The clear relationship between LANCL1 and bacterial LanC proteins suggests similar functions as peptide-modifying enzymes synthesizing antimicrobial peptides. In particular, the high expression of LANCL1 in testis and brain, organs separated by blood-tissue barriers, may hint at a role in the immune surveillance of these organs.

Keywords: Lantibiotic; p40; GPR69A; Neuron; *Rattus* spp

1. Introduction

The 40 kDa protein LANCL1 (alias p40/GPR69A) was originally isolated from human erythrocyte membranes (Mayer et al., 1998b) by using its affinity for the C-terminus of the membrane protein stomatin (band 7.2b). It is mainly expressed in the brain, testis, various epithelia, and diverse hematopoietic cells (Mayer et al., 1998a,b). Sequence analyses showed that human and mouse LANCL1 contain seven hydrophobic stretches with predicted α-helical transmembrane structure (Mayer et al., 1998a,b), thereby suggesting a G protein-coupled receptor structure and function. However, recent data revealed that LANCL1 is a peripheral membrane protein similar to the bacterial lanthionine synthetase C (LanC) components (Bauer et al., 2000), which are part of a multimeric membrane-associated complex involved in the modification and transport of peptides (Siegers et al., 1996; Kiesau et al., 1997). The produced peptides, termed lantibiotics (Sahl and Bierbaum, 1998), have antimicrobial properties. Functionally similar defense peptides exist in mammals and other vertebrates, insects, and plants (Ganz and Lehrer, 1998; Borregaard et al., 2000). Homologs of LANCL1 are present in *Drosophila melanogaster*, *Arabidopsis thaliana*, and *Solanum chacoense*, indicating that this protein plays a fundamental role in animals and plants. In the present study we describe the cloning, characterization and tissue-specific expression of rat LANCL1, a new member of the growing family of eukaryotic LanC-like proteins.

2. Materials and methods

2.1. cDNA cloning and sequence analysis

Rat cDNA clones were identified by BLAST (Altschul et al., 1990) searches of the dbEST database. Selected clones were then obtained from Research Genetics (Huntsville, AL) or ATCC (American Type Culture Collection, Manassas, VA) and characterized by standard methods

Abbreviations: bp, base pair(s); cDNA, DNA complementary to RNA; EST, expressed sequence tag; kb, kilobase(s) or 1000 bp; LANCL, LanC-like protein; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; UTR, untranslated region

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(Sambrook et al., 1989). 5′- and 3′-RACE PCR was performed using a Marathon-Ready™ cDNA library from rat brain (Clontech, Palo Alto, CA) and the Advantage™ cDNA PCR Kit (Clontech) according to the instructions of the manufacturer. We designed and used two gene-specific primers according to an overlapping EST (GenBank accession number AA818760) sequence (Fig. 1). The primer P1 (5′-GCCTCAACTGTTTGAGCAGGCGGTC-3′) was used for 3′-RACE and the primer P2 (5′-ATCCTCTGC-CTGCTTCCCGCTGTTC-3′) for 5′-RACE in combination with the Marathon anchor primers. The PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) and characterized by standard methods (Sambrook et al., 1989). The nucleotide sequence compilation, deduction of the amino acid sequence, and database searches were accomplished by the use of the GCG (Genetics Computer Group, Madison, WI) software package available on EMBnet.

2.2. Multiple sequence alignment

BLAST (Altschul et al., 1990) searches in the non-redundant database identified sequences belonging to the eukaryotic LanC-like protein family. The corresponding amino acid sequences were aligned by CLUSTALW (Thompson et al., 1994), and shaded by the program BOXSHADE. The value for the fraction of the sequences that must agree for shading was set to 0.3. Identical residues are highlighted as white text on black background, similar residues are shown as white text on grey background.

2.3. Northern blot analysis

Northern blot analysis of multiple rat tissues (Clontech MTN™) was performed according to the instructions of the manufacturer. A rat LANCL1-specific cDNA clone (3′-RACE PCR product named ISHP in Fig. 1) was purified, ^{32}P-labeled using the Multiprime DNA labeling system from Amersham (UK), and hybridized in ExpressHyb (Clontech) solution. In addition, the blot was probed with a β-actin control cDNA (Clontech). The intensities of the obtained bands were measured as counts per minute using a quantitative program of an Instant Imager (Packard, Meriden, CT).

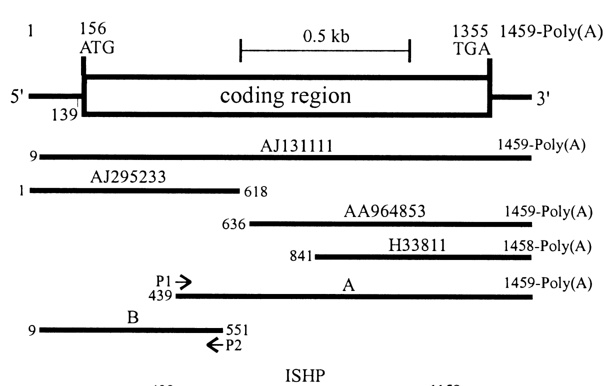

Fig. 1. Schematic model of the rat LANCL1 cDNA. The coding region is depicted by the enclosed box with the positions of the initiation codon (ATG) and the termination codon (TGA) shown. 5′- and 3′-UTRs are indicated by lines. The cloning and sequences of AJ131111 and AJ295233 (GenBank accession numbers) are described in the text. The relative positions of two characterized EST clones are shown below. 3′- and 5′- RACE PCR clones are denoted A and B, respectively. The corresponding gene-specific PCR-primers are named P1 and P2. Additionally, the position of the cRNA probe used for in situ hybridization analyses is indicated (ISHP). Numbering of nucleotides is from 5′ to 3′.

2.4. Western blot analysis

A rat multiple tissue blot and brain blot (Chemicon International, Inc., Temecula, CA) containing 75 and 100 μg of total protein per lane, respectively, was probed with the H22 antibody (Bauer et al., 2000). This antibody specifically reacts with the LANCL1 N-terminus. In general, the analysis was performed as described (Snyers et al., 1998), except that anti-rabbit IgG-HRP (Pierce, Rockford, IL) was used as the secondary antibody.

2.5. In situ hybridization analysis

Two different methods have been employed for in situ hybridization analysis of rat tissues. The size and relative position of the rat LANCL1-specific cDNA clone used for in vitro transcriptions are indicated in Fig. 1 (ISHP). This truncated 3′-RACE PCR fragment was cloned into the pCRII-TOPO vector (Invitrogen). Flanking T7 and Sp6 promoters allowed the in vitro transcription of antisense and sense RNA probes following vector linearization. The first method used radioactively labeled riboprobes transcribed from the ISHP clone. Cryosections (7 μm thick) of rat testis, liver, and heart were mounted onto silanated slides (ORION Molecular Services, Manchester, UK). The linearized ISHP clone was subjected to in vitro transcription in order to produce radioactively labeled antisense (2.8×10^6 cpm) and sense (2.5×10^6 cpm) riboprobes, both were used for hybridization. Exposure time was set to 7, 10, and 14 days. Photographic development produced silver grains over the probe-positive cells of interest. The sections were counterstained with hematoxylin and eosin.

The second method was used for probing cryosections (6 μm thick) of rat cerebellum.

In vitro transcriptions were carried out using the DIG RNA Labeling Kit (Boehringer Mannheim, Germany), unincorporated nucleotides were removed with a QIAquick Nucleotide Removal Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturers. Aliquots were analyzed for the size of the RNA. The amount of incorporated digoxigenin-labeled UTP was determined by the use of an anti-DIG antibody (Boehringer Mannheim). Both transcripts gave concentrations of about 4 ng/μl. The probes were stored at −80°C. Prehybridization, hybridization and washing steps were carried out using the SureSite II Hybridization Reagents Kit (Novagen, Madison, WI).

Hybridization was performed at 0.5 ng/μl RNA probe and 52°C. The probe was detected by the anti-DIG antibody (Boehringer Mannheim) conjugated with alkaline phosphatase (NBT/BCIP-staining). Slides were mounted using Aquatex (Merck, Darmstadt, Germany), a water-based mounting solution. Photomicrographs were taken using a Nikon Mikrophot-FXA-microscope.

3. Results and discussion

3.1. Characterization of the rat LANCL1 cDNA

Using the predicted mouse LANCL1 amino acid sequence (Y16518) in a TBLASTN search (Altschul et al., 1990) of the dbEST database, several EST clones coding for the LANCL1 ortholog from rat were detected. The sizes and the positions of selected EST clones are shown in Fig. 1. The clone AA9648534 was derived from adult muscle tissue of Sprague–Dawley rats, whereas H33811 was isolated from rat PC-12 cells, which had been treated with nerve growth factor for 9 days. For RACE analysis of a rat brain cDNA library, two oligonucleotide primers were designed. The 3′ RACE, performed with primer P1 and the anchor primer, yielded two different fragments, at 1 and 0.7 kb. The shorter fragment (termed ISHP in Fig. 1) turned out to be an abnormally truncated cDNA, while the longer one (‘A’ in Fig. 1) included the full 3′ sequence and a poly(A) tail. Like in the murine transcript, the polyadenylation site is preceded by an imperfect polyadenylation signal (AATGAA). The 5′ RACE performed with primer P2 yielded a fragment of 0.5 kb (‘B’ in Fig. 1) including the predicted start methionine and part of the 5′-UTR. The sequence information of the RACE PCR clones was combined and submitted to GenBank (accession number AJ131111). Additionally, the complete insert of EST clone AA818760 was sequenced and submitted under the accession number AJ295233 (containing eight additional bases at the 5′- end).

The analysis of the combined sequences revealed an open reading frame of 1352 bp and a coding region of 1197 bp (399 amino acids), starting from the first ATG. The start codon is located within an appropriate consensus sequence for the initiation of translation (Kozak, 1984). Unfortunately, none of the analyzed rat clones covered the complete 5′ UTR corresponding to the mouse full-length LANCL1 cDNA sequence (AJ294535). The 5′-UTR sequence starts within an unspliced intron, which is also present in the majority of the mouse LANCL1 cDNAs (Mayer et al., 1998a), but lacks the 5′ splice site of the intron. The 3′ splice site is located at position 139 of the composite sequence (Fig. 1). Interestingly, all the rat clones analyzed so far contain this unspliced intron. This finding indicates that the intron-containing type of the LANCL1 transcripts is the major one in mouse and rat. Remarkably, the intron sequence is highly conserved between these species (88.6%), suggesting that this intron might have important regulatory functions. However, the low degree of similarity with the human intron (about 55% identity) present at this position in the genomic DNA, but not in the mRNA, suggests that this function might be confined to the rodent lineage.

3.2. Comparison of rat LANCL1 with other members of the LanC-like protein family

The deduced amino acid sequence of rat LANCL1 shows a high degree of homology when compared with the human (91.5% identity, 95.2% similarity) and murine (96% identity, 97.7% similarity) sequences. All seven hydrophobic segments containing the described G-X-X-G motif (Mayer et al., 1998b; Bauer et al., 2000) are absolutely identical between rat and mouse LANCL1 and all 13 cysteine residues are invariable (Fig. 2). From *A. thaliana*, two sequences are known, one is more related to LANCL1 and the other to LANCL2, as indicated. The *D. melanogaster* and *S. chacoense* proteins show rather equal similarities to both LANCL1 and LANCL2. In prokaryotes, the G-X-X-G motif is not strictly conserved in LanC and LanM proteins, whereas several motifs already described (Meyer et al., 1995; Kupke and Gotz, 1996) are invariable, notably HG in segment 4, WCXG in segment 5 and CHG in segment 6. These residues are also conserved in all known eukaryotic LanC-like proteins, except for the *Solanum* protein (Fig. 2). The cysteine residues in segments 5 and 6 (marked by ‘C’ in Fig. 2) were suggested to play a role as active sites of LanC enzymes (Kupke and Gotz, 1996). Two conserved glycine residues in segments 2 and 4 (marked by ‘G’ in Fig. 2) were shown by mutational analysis to be essential for the function of the LanC protein EpiC (Kupke and Gotz, 1996). However, it is not known whether these residues are active sites of the enzyme or binding sites for coenzymes or whether they are only structurally significant. The high degree of evolutionary conservation within the LanC-like protein family, notably within the seven hydrophobic repeats, suggests that these proteins play a fundamental role in animals and plants.

3.3. Northern blot analysis of LANCL1 mRNA in rat tissues

Northern blot analysis of rat multiple tissues revealed the presence of a major 1.5 kb transcript highly expressed in testis, and a 5 kb transcript most abundant in brain (Fig. 3). Interestingly, an additional band at 1.1 kb is present in skeletal muscle, raising the possibility of an alternatively spliced LANCL1 product in this tissue. For comparison, there are two different LANCL1 transcripts detectable on mouse Northern blots (1.7 and 5 kb) (Mayer et al., 1998a), resulting from alternative polyadenylation (GenBank accession number AJ294535), and also two isoforms of human LANCL1 (1.7 and 4.8 kb), produced by the same mechanism (Mayer et al., 1998b). However, in man the 4.8 kb isoform is the major transcript in all positive tissues, notably the brain and testis. It is therefore likely, that the 5 kb isoform of rat LANCL1 also arises from alternative polyadenylation. Although Northern blots are not suitable for accurate quantitative measurements, a normalization procedure using an actin control probe can nevertheless shed some light on the tissue-specific expression levels. In the case of rat LANCL1 there is an obvious high expression in

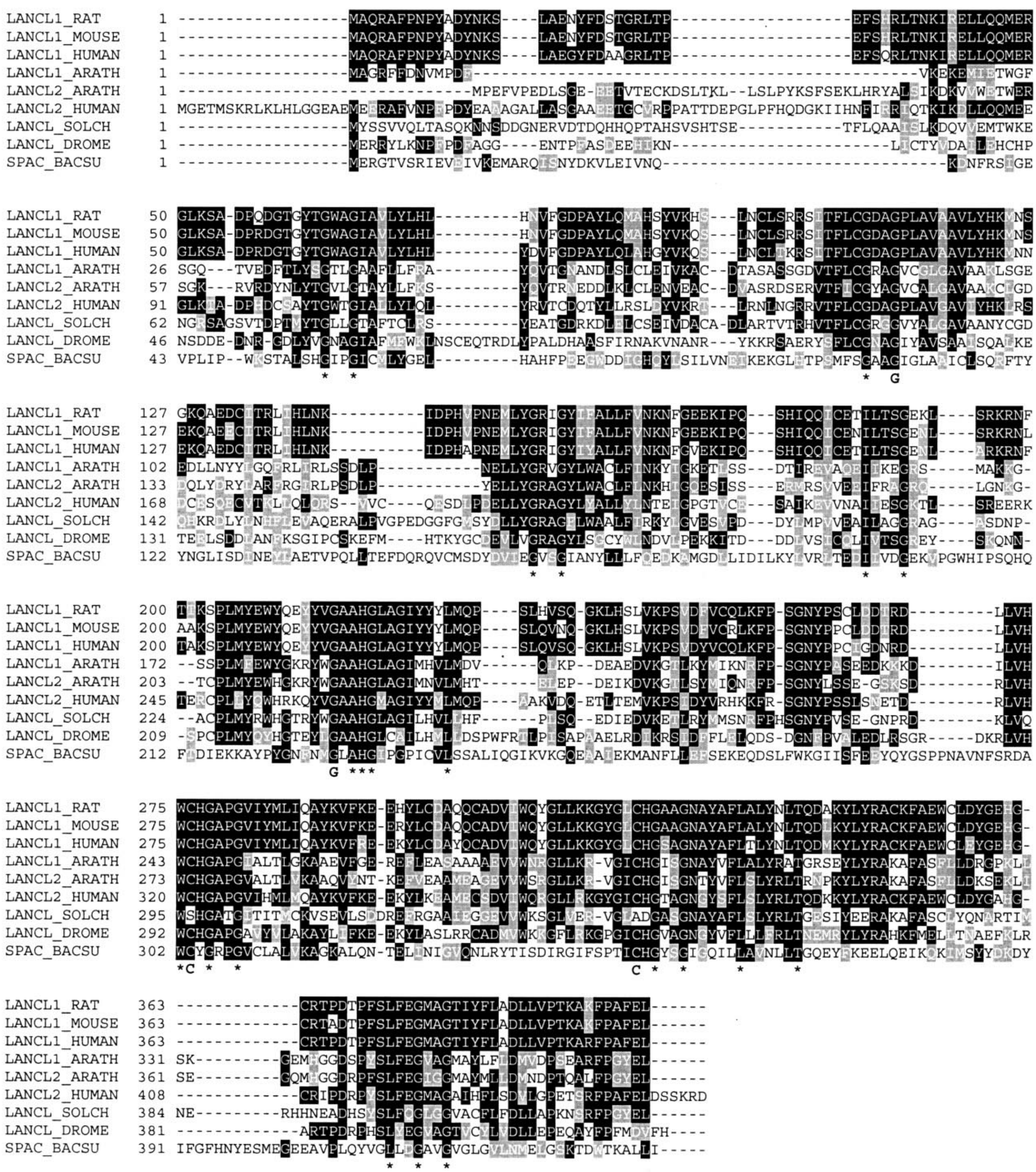

Fig. 2. Amino acid sequence of rat LANCL1 and multiple alignment with other members of the LanC-like protein family and one prokaryotic LanC protein, SpaC. The amino acid sequences were aligned by CLUSTALW and shaded by BOXSHADE. Identical residues are highlighted as white text on black background, similar residues are shown as white text on grey background. Completely conserved residues are marked by asterisks. Two conserved glycine residues which are essential for the function of the LanC protein EpiC from *Staphylococcus epidermidis* are labeled ‘G’. Two cysteine residues suggested as active sites of LanC enzymes are labeled ‘C’. The proteins aligned are (name, species and GenBank or Swissprot accession numbers in parentheses): LANCL1_RAT (rat LANCL1, AJ131111), LANCL1_MOUSE (mouse LANCL1, Y16518), LANCL1_HUMAN (human LANCL1, Y11395), LANCL1_ARATH (*A. thaliana* AAD20918), LANCL2_ARATH (*A. thaliana* AAF30340), LANCL2_HUMAN (human LANCL2, AJ278245), LANCL_SOLCH (*S. chacoense* AF272710), LANCL_DROME (*D. melanogaster* AAD38664), SPAC_BACSU (*Bacillus subtilis* P33115).

testis, notably of the short transcript, about 20 fold relative to brain. In contrast, the 5 kb isoform is about three times more abundant in brain than in testis. As a conclusion, we may therefore suggest tissue-specific regulatory functions within the long 3′-UTR.

Fig. 4. Immunochemical analysis of rat LANCL1 tissue distribution. A rat multiple tissue blot (A) and brain blot (B) were probed with an antibody directed against the N-terminus of LANCL1. A single band of 40 kDa is present in all lanes. (A) LANCL1 is expressed in all tissues analyzed at varying amounts. The highest level of expression is found in testis and brain. (B) LANCL1 is expressed throughout various parts of the rat central nervous system. The strongest bands appear in cortex and cerebellum whereas the weakest signal is detected in spinal cord.

3.4. Immunochemical analysis of rat LANCL1 tissue distribution

The high level of expression of rat LANCL1 mRNA in testis and brain was nicely confirmed by Western blot analysis showing prominent 40 kDa LANCL1 bands in these tissues (Fig. 4A). In addition, a basal level of expression was revealed in all of the tissues after longer exposure time. However, the signals in heart and liver are obviously weaker compared to those on the Northern blot (Fig. 3). LANCL1 is highly expressed throughout various parts of the rat central nervous system (Fig. 4B), notably in cortex and cerebellum, similar to man (Mayer et al., 1998b). The weak signal in spinal cord, as compared to the Northern analysis, may reflect a relatively lower LANCL1 protein content or a higher turnover.

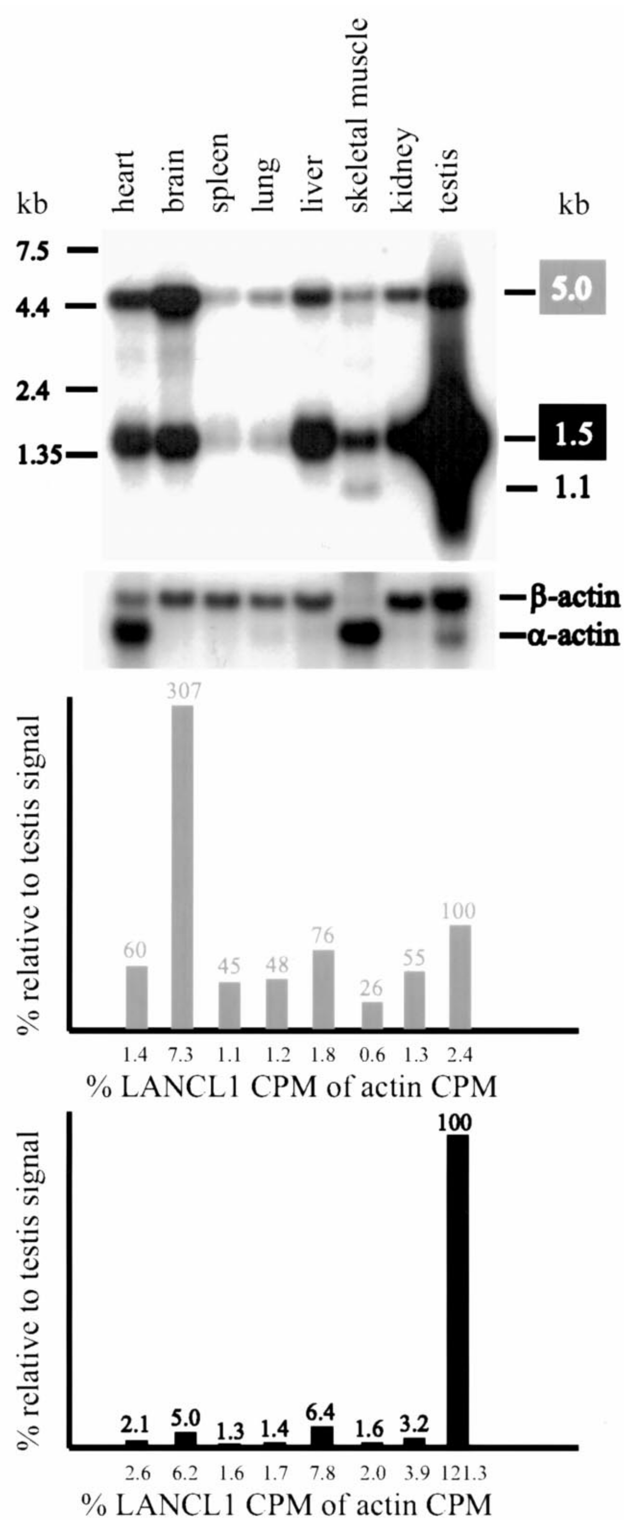

Fig. 3. Rat LANCL1 multiple tissue Northern blot analysis. Upper part: a blot containing 2 μg poly(A)$^+$ RNA per lane was hybridized with a ^{32}P-labeled rat LANCL1 cDNA probe and autoradiographed. Hybridization with a β-actin probe as a standard is shown below. RNA size markers are depicted on the left side, the sizes of specific bands are given on the right side. Middle part: the signals of the 5 kb transcript are normalized to the expression levels of the β-actin probe (in heart and skeletal muscle, the α-actin signal was used for comparison) and then compared relatively with the testis signal. Lower part: the same procedure was applied for the 1.5 kb transcripts. The 1.5 kb transcript is massively expressed in testis, whereas the 5 kb transcript is most abundant in brain. In skeletal muscle, an additional band at 1.1 kb is visible, possibly representing an alternative splicing product.

3.5. In situ hybridization analysis of rat LANCL1

In situ hybridization analysis was performed in order to identify the cell types responsible for the pronounced expression of rat LANCL1 in some tissues, especially testis and brain. Two different labeling methods were employed, a radioactive and non-radioactive one (see section 2.5.). Strong signals were observed in the germinal cells of the seminiferous tubules of testis (Fig. 5A,B), in the Purkinje cells and neurons of the granular layer of the cerebellum

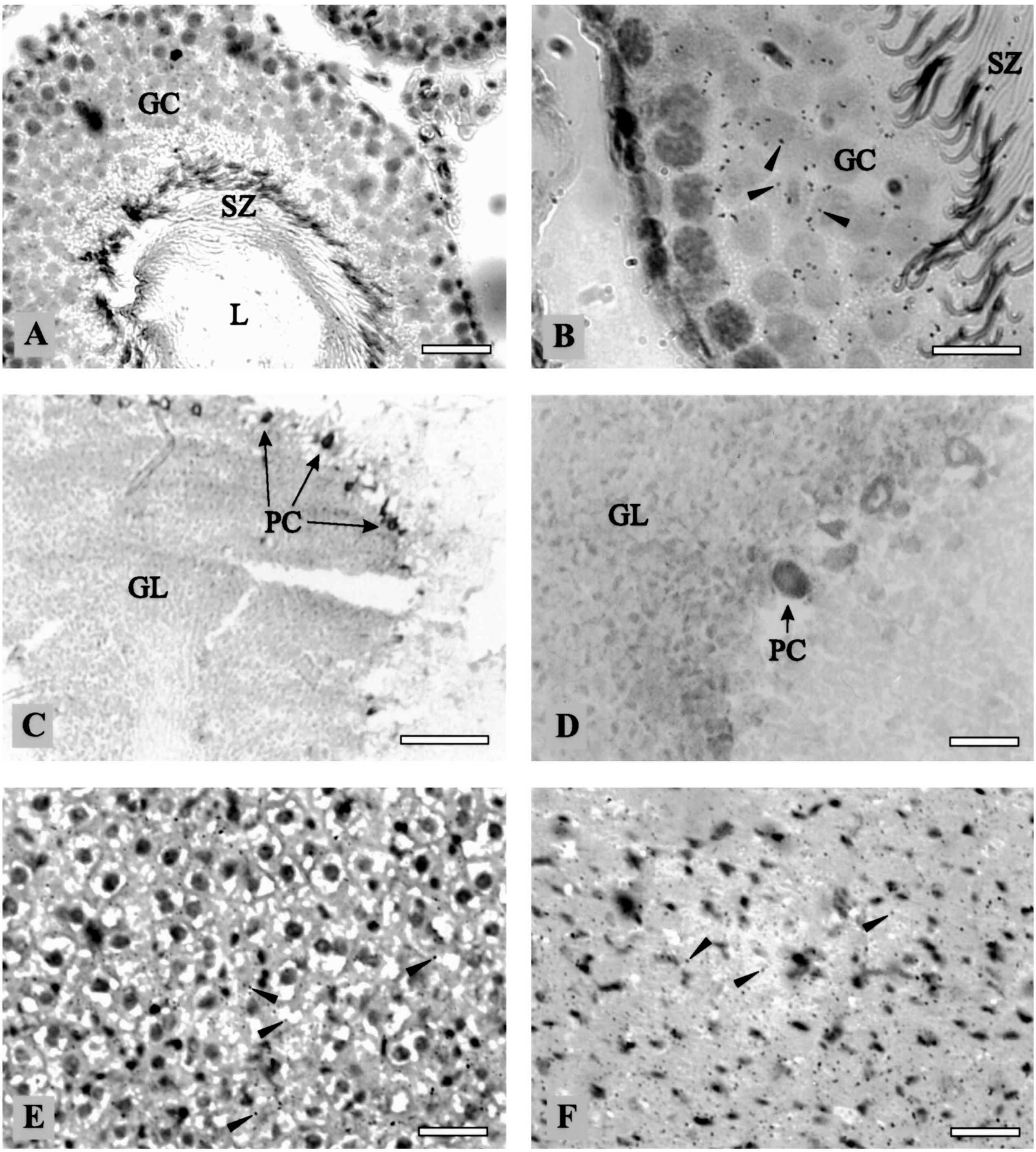

Fig. 5. In situ hybridization analysis. Fixed cryosections of rat tissues were hybridized with a radioactively labeled (A,B,E,F) or digoxigenin-labeled (C,D) rat LANCL1-specific cRNA probe. Strong signals were observed in the germinal cells (GC) of the seminiferous tubules of testis (A,B), in the Purkinje cells (PC) and neurons of the granular layer (GL) of cerebellum (C,D), in hepatocytes of the liver (E) and myocytes of the heart (F). The radioactive method produced silver grains which are marked by arrowheads. The DIG-method produced a staining of the whole cytoplasm in positive cells. SZ, spermatozoa; L, lumen of the seminiferous tubules. Scale bar: 400 μm (C), 100 μm (A, D–F), 40 μm (B).

(C,D), in hepatocytes of the liver (E) and myocytes of the heart (F). In testis, each seminiferous tubule has a central lumen lined by an actively replicating epithelium composed of the germinal cells and a population of supporting cells, the Sertoli cells. Spermatogenesis occurs along the length of the tubules, thus showing cells at several maturation stages in a cross-section. In Fig. 5B, maturation proceeds from the dark staining spermatogonia on the left side to the spermatozoa on the right, which are finally released into the lumen. LANCL1 expression in Purkinje cells and neurons of the granular layer is consistent with previous observations in rhesus monkey and mouse sections (Mayer et al., 1998a,b). Hepatocytes and cardiac myocytes have not been identified before in the context of LANCL1 expression.

Various mammalian defense peptides have been shown to have a broad tissue distribution, like porcine beta-defensin-1, which is expressed in brain, testis, liver and heart, among other tissues (Zhang et al., 1998). Mouse testicular cells synthesize antimicrobial peptides related to the defensins of the cryptdin family (Grandjean et al., 1997). These peptides were first identified in intestinal crypt cells, which interestingly also highly express LANCL1 in mouse (Mayer et al., 1998a). Inside the seminiferous tubules, these cryptdins were found accumulated in Sertoli cells. As these cells are poorly defined in light microscopic sections, it may easily be possible that at least part of the LANCL1 signal seen in Fig. 5B comes from this cell type. Recently, an antimicrobial peptide was described in the male reproductive system of rats showing similarity with beta-defensins (Li et al., 2001). In the light of these data, it is conceivable that LANCL1 could play a role in the native immune defense systems of tissues like testis and brain, which are organs separated by blood-tissue barriers.

3.6. Conclusions

1. We isolated and characterized the cDNA coding for the rat lanthionine synthetase component C-like protein 1 (LANCL1). The deduced amino acid sequence showed 91.5% and 96% identity when compared with the previously characterized human and mouse orthologs, respectively. Bacterial LanC proteins are involved in the thioether formation during the biosynthesis of lantibiotic peptides. We conclude that rat LANCL1 is a novel member of the eukaryotic LanC-like protein family and suggest that it has a LanC-related function as part of a membrane-associated peptide-modifying enzyme complex.
2. Northern blot analysis of rat tissues showed the presence of a 1.5 kb LANCL1 transcript highly expressed in testis and a 5 kb transcript most abundant in brain. Comparison with the human and mouse mRNA isoforms suggests that these transcripts originate from alternative polyadenylation. We conclude that brain-specific elem-ents probably reside in the distal 3′-UTR. The high level of expression of rat LANCL1 in testis and brain was confirmed by Western blotting.
3. In situ hybridization analyses of various rat tissues revealed a strong signal in the germinal cells of the seminiferous tubules in testis, in the neurons of the cerebellum, in liver hepatocytes, and in cardiac myocytes. The clear relationship between LANCL1 and bacterial LanC proteins suggests similar functions as peptide-modifying enzymes. We conclude that the pronounced expression of LANCL1 in testis and throughout the whole brain, organs separated by blood-tissue barriers, might be important for the native immune defense system of these tissues.

Acknowledgements

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