Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver

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Abstract

The human genome contains numerous genes whose protein products are unknown in terms of structure, interaction partner, expression, and function. To unravel the function of these orphan genes, it is of particular value to isolate native forms of protein and peptide products derived from these genes. From human blood ultrafiltrate, we characterized a novel gene-encoded, cysteine-rich, and cationic peptide that we termed liver-expressed antimicrobial peptide 2 (LEAP-2). We identified several circulating forms of LEAP-2 differing in their amino-terminal length, all containing a core structure with two disulfide bonds formed by cysteine residues in relative 1–3 and 2–4 positions. Molecular cloning of the cDNA showed that LEAP-2 is synthesized as a 77-residue precursor, which is predominantly expressed in the liver and highly conserved among mammals. This makes it a unique peptide that does not exhibit similarity with any known human peptide regarding its primary structure, disulfide motif, and expression. Analysis of the LEAP-2 gene resulted in the identification of an alternative promoter and at least four different splicing variants, with the two dominating transcripts being tissue-specifically expressed. The largest native LEAP-2 form of 40 amino acid residues is generated from the precursor at a putative cleavage site for a furin-like endoprotease. In contrast to smaller LEAP-2 variants, this peptide exhibited dose-dependent antimicrobial activity against selected microbial model organisms. LEAP-2 shares some characteristic properties with classic peptide hormones and it is expected that the isolation of this novel peptide will help to unravel its physiological role.

Keywords: Alternative splicing; antimicrobial activity; disulfide bonds; hemofiltrate; liver; peptide; secretion

As a consequence of the efforts to sequence and assemble the human genome (Lander et al. 2001; Venter et al. 2001), the systematic analysis of peptides and proteins as the functional gene products produced by a given cell population or tissue under defined conditions is considered to be the next milestone in molecular biology. The estimated number of genes is unexpectedly low, and the number of biologically active peptides and proteins cannot be deduced from these data because of events such as alternative splicing of mRNA precursors, usage of alternative gene promoters, pseudogenes, and alternatively processed proteins. The number of proteins is therefore estimated to be two to three orders of magnitude higher than the number of ~40,000 genes annotated in the human genome (Harrison et al. 2002; Rappsilber and Mann 2002). Therefore, the mass-spectrometric identification of a gene product in proteomics or, even better, the isolation of novel proteins and peptides followed by a struc-

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Abbreviations: CFU, colony-forming unit; ESIMS, electrospray ionization mass spectrometry; LEAP, liver-expressed antimicrobial peptide; RT-PCR, reverse transcription/polymerase chain reaction; TFA, trifluoroacetic acid.

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tural analysis in terms of primary structure and posttranslational modifications are of increasing importance. In particular, it is known for polypeptides such as hormones and cytokines that the mature bioactive molecular form cannot be deduced from the corresponding genes (Zadina et al. 1997; Wuyts et al. 1999).

The systematic analysis of a set of peptides and proteins of any biological origin represents a significantly different challenge compared with genome sequence analysis. To approach this, our laboratory has focused on the systematic analysis of human blood polypeptides with a molecular weight of <20 kD. The technology we established is a large-scale human peptide library produced by chromatographic processing of human blood ultrafiltrate (Schulz-Knappe et al. 1997). This library was successfully screened using specific bioassays and high-affinity receptor ligands were identified (Detheux et al. 2000). In addition, the peptide library was of particular value for the isolation of polypeptides by chromatographic purification to homogeneity without biological testing. This approach, termed peptide trapping, led to the identification of numerous peptides and their molecular forms, thereby enabling a subsequent functional characterization, for example, for the serine protease inhibitor LEKTI (Mägert et al. 1999), the antimicrobially active peptide LEAP-1/hepcidin (Krause et al. 2000), the angiogenesis inhibitor endostatin (John et al. 1999), and the uPAR-related polypeptide SLURP-1 (Adermann et al. 1999).

In this communication we report the isolation and biochemical characterization of a novel peptide we have termed LEAP-2 (liver-expressed antimicrobial peptide). To identify the peptide, we initially used a mass spectrometry-based technique to search for cysteine-rich peptides, as these often display important biological activities (Sillard et al. 1993). LEAP-2 is the second blood-derived peptide that is expressed predominantly in the liver and exhibits antimicrobial activity. As the first blood-derived antimicrobial peptide from the liver, LEAP-1/hepcidin (Krause et al. 2000; Park et al. 2001) has been reported to be involved in iron homeostasis (Nicolas et al. 2001; Pigeon et al. 2001); important physiological functions might be possible for LEAP-2 as well.

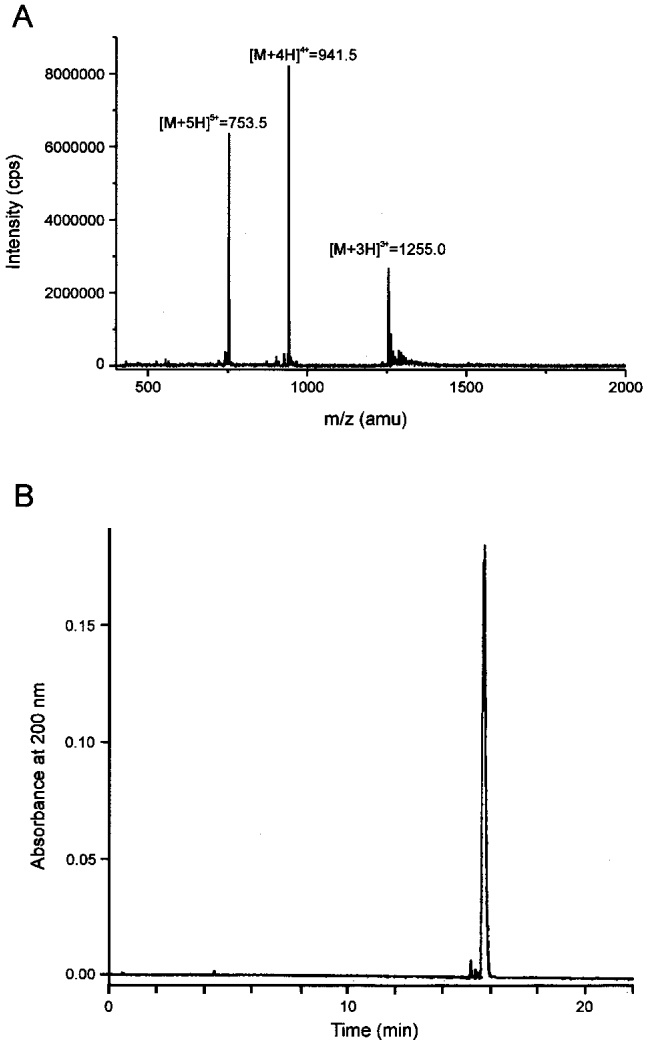

Figure 1. Electrospray mass spectrometric and capillary zone electrophoretic analysis of purified LEAP-2. The peptide initially purified from human blood hemofiltrate had a molecular mass of 3762.1 Da (*A*), corresponding accurately to the sequence positions 44–77 of the LEAP-2 precursor (the three- to fivefold protonated molecular ions of LEAP-2 were detected by electrospray mass spectrometry). Isolated LEAP-2-(44–77) was highly homogenous as demonstrated by capillary zone electrophoresis (*B*).

Results

Identification of LEAP-2 peptides

The first LEAP-2 variant was isolated from a human hemofiltrate peptide library by four subsequent chromatography steps. The purity and identity of the peptide were determined by capillary zone electrophoresis and ESIMS analysis (Fig. 1). Edman degradation resulted in the sequence of LEAP-2-(44–77) (Fig. 2), with blank cycles for the four cysteine residues. The molecular mass found by ESIMS (M_r = 3762.1) is in full agreement with the mass of the peptide assuming cysteine residues for the positions corresponding to the blank cycles. On limited proteolysis of this peptide with trypsin, two fragments were separated and characterized by ESIMS and then sequenced. The primary structures determined for these branched peptides were GVSLRPIGASC(LCR)R (M_r = 1603.3) and DDSEC(CSL SVAQE)ITR (M_r = 1770.9). Therefore, the disulfide connectivity of LEAP-2 is Cys^{54}–Cys^{65} and Cys^{60}–Cys^{70}.

During our comprehensive investigations, to systematically search for novel circulating human peptides, we identified LEAP-2-(38–77) as the largest peptide. In addition, LEAP-2 peptides corresponding to the precursor positions

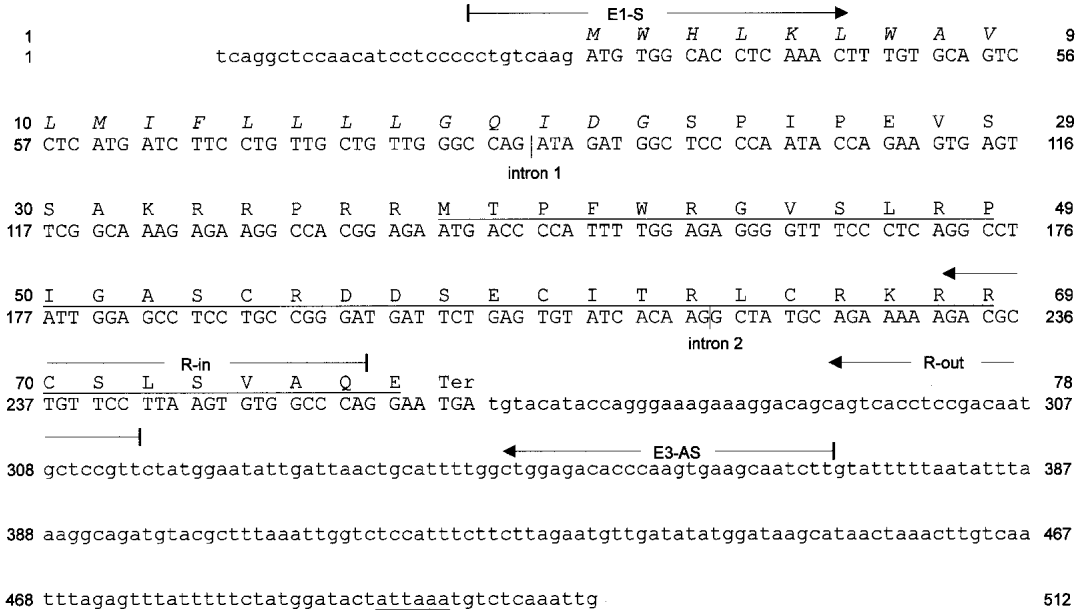

Figure 2. Human LEAP-2 precursor cDNA and deduced amino acid sequence. Coding regions are printed in capital letters. The typical secretory signal sequence 1–22 is printed in italics, whereas the positions of introns 1 (198 bp) and 2 (320 bp) of the LEAP-2 gene are marked by vertical lines. Two TATAAA sequences located 31 and 84 bases upstream of the translational start were identified. The putatively mature and antimicrobially active LEAP-2-(38–77) isolated from hemofiltrate and the putative polyadenylation signal are underlined. The position of the primers used for nested 5′-RACE-PCR and for preparative PCR from six different species is indicated.

39–77, 43–77, and 48–77 were identified. As a second group of LEAP-2 peptides, carboxy-terminally truncated derivatives corresponding to the precursor positions 48–73, 48–75, 46–76, and 48–76 were found. These peptides were characterized by ESIMS and sequencing, demonstrating that they contain an intact core structure with two disulfide bonds. Interestingly, a 1877-Da metabolite of LEAP-2 corresponding to the sequence positions 44–61 was identified later by ESIMS and sequencing from a human urine peptide library of healthy donors.

Identification and characterization of the LEAP-2 cDNA

Scanning the LEAP-2 primary structure obtained experimentally against protein databases revealed that neither this peptide nor any significantly related sequences have been reported so far. Using the BLAST (tblastn) nucleic acid alignment program, we identified a genomic sequence from chromosome 5 (GenBank accession number AC004500), which is related to LEAP-2. In addition, we could assign several human (e.g., T60528, R11462), murine (W29825, AA238147), and porcine (BE233297, BF078725) EST clones to LEAP-2. Analyzing the overlapping sequences of these liver-derived clones as well as the genomic sequence from clone AC004500, we concluded that the LEAP-2 gene consists of three exons and two introns. The complete cDNA sequence encoding a 77-amino acid residue precursor protein and the deduced primary structure of LEAP-2 are shown in Figure 2.

Standard PCR with *LEAP-2*-specific primers E1-S and E3-AS (Fig. 2) was carried out to confirm the database entry. A pattern of five distinct bands was obtained, depending on the origin of the template cDNA. The main PCR product obtained from liver, kidney, small intestine, colon, gastric antrum and bladder was a 350-bp band, whereas all other tissues tested mainly expressed a 550-bp transcript. Another difference between the tissues mentioned is that their analysis resulted in either a band at 670 bp or at 720 bp (Fig. 3A). Subsequent cloning and sequencing of all bands revealed the existence of at least three splicing variants of the LEAP-2 gene apart from the completely spliced cDNA, namely an intron 1-retaining form *LEAP-2*^{550}, an intron 2-retaining form *LEAP-2*^{670}, and a cDNA that contained both intronic sequences (*LEAP-2*^{860}) (Fig. 2). Except for *LEAP-2*^{670}, which was mainly found in tissues simultaneously expressing the completely processed *LEAP-2*^{350} as major transcript (liver, kidney, duodenum, jejunum, bladder, and brain), all splicing variants are represented in the human EST division at GenBank (e.g., accession number AV650047 related to *LEAP-2*^{860}, T60528 to *LEAP-2*^{350}, and AF143867 to *LEAP-2*^{550}). Four of the *LEAP-2*-specific EST clones published to date (AI302776, AI590215, AI394524, AI948486) are derived from cancerogenic tissues of different cellular origin.

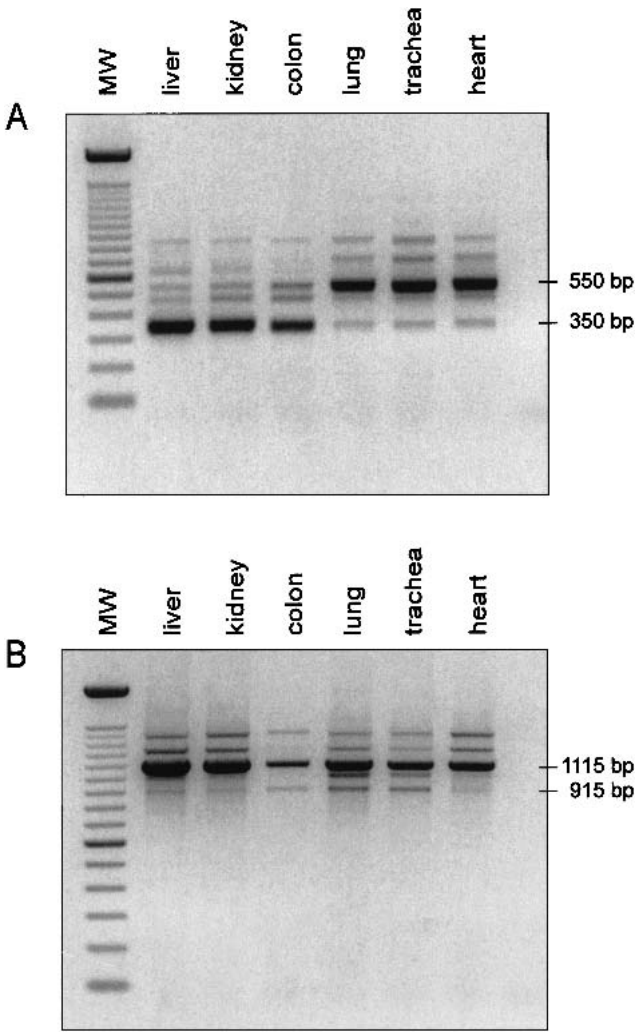

Figure 3. Alternative splicing in the LEAP-2 gene. (*A*) Standard PCR conducted with the primer combination E1-S and E3-AS (Fig. 2) and 20 ng of DNase-treated cDNA. *LEAP-2*^{350} is mainly expressed in liver, kidney, and colon, whereas *LEAP-2*^{550} is the main transcript in lung, trachea, and heart. PCR products at 650 bp, 720 bp, and 870 bp represent splicing variants of *LEAP-2*, and the band at 480 bp could be identified as a PCR artefact. (MW = marker, 100 bp DNA ladder, Life Technologies). (*B*) Standard PCR performed with the primers PROM-S (5′-GGTGCA GATTAGGGTGACAGTCCATC-3′), which is located 565 bp upstream of the transcriptional start depicted in Figure 2, and E3-AS. Lung, heart, and trachea exhibit the same pattern of bands, including the 565 bp shift in size caused by the upstream primer PROM-S. The main transcript identified in liver, kidney, and colon, however, changes from the completely spliced *LEAP-2*^{350} to the intron 1-retaining *LEAP-2* variant. All bands obtained were characterized by DNA sequencing.

Chromosomal localization of the LEAP-2 gene

Using radiation hybrid mapping, the LEAP-2 gene was located on chromosome 5q31, ~1.6 cR proximal to the STS-marker WI-7743, which corresponds to a partial sequence of the interleukin-13 gene. These data are supported by the nucleic acid sequence of transforming growth factor β, which is located in 10 kb distance to the LEAP-2 gene on contig AC004500 and which can be assigned to human chromosome 5q31 using the NCBI map viewer (http://www.ncbi.nlm.nih.gov:80/cgi-bin/Entrez/map_search). A BLAST search revealed a *LEAP-2*-containing contig derived from mouse chromosome 11 (AC011013), thereby confirming the formerly found synteny between mouse chromosome 11 and human chromosome 5q31 (Wenderfer et al. 2000; Lander et al. 2001).

Detection of an alternative promoter

Size heterogeneity observed for LEAP-2-specific mRNA detected by Northern blot (Fig. 4) as well as by the EST clone AF143867, which contained a prolonged 5′-region compared with the LEAP-2 form originally isolated from liver, indicated the existence of a LEAP-2 mRNA isoform. Nested 5′-RACE-PCR using cDNA obtained from different tissues (Human Marathon-Ready cDNA, Clontech) resulted in a single PCR product in liver (Fig. 2), whereas multiple LEAP-2-specific bands were obtained with the corresponding kit from human lung. These findings, however, are in contrast to the results obtained by Northern blot analysis, where at least two LEAP-2-specific signals were detected in the liver. In this experiment, a ^{32}P-labeled LEAP-2-specific cDNA fragment did not hybridize with poly(A$^+$) RNA from lung (Fig. 4).

The longest RACE-PCR product obtained from human lung contained an additional 1345 bp upstream of the 5′-end of the liver-derived RACE-PCR product. This cDNA of 1.8 kb, which is located 76 bp downstream of a TATATA sequence and whose size corresponds to the 2-kb signals detected by Northern blot (Fig. 4), could be amplified by subsequent standard PCR in all tissues tested. PCR with primers located in the upstream sequence revealed that the distal promoter, in contrast to the proximal promoter, preferentially generates a transcript that includes intron 1 (Fig. 3B). Moreover, additional PCR analysis carried out with an intron 2-specific primer demonstrated that the distal promoter does not code for a transcript that includes the former second intron. Despite the differing results obtained from Northern blot analysis and RACE-PCR, the detection of the above-mentioned cDNA variants in all tissues tested reveals the existence of at least one alternative starting point of transcription. The significance of the alternative 5′-untranslated region remains to be elucidated.

Sequence similarity in different species

The above-mentioned EST clones from mouse were used to search for murine homologs of the LEAP-2 gene. The radioactively ^{32}P-labeled PCR products of two primers deduced from these EST sequences served as probes to screen a cosmid library derived from the mouse line 129/Sv. Com-

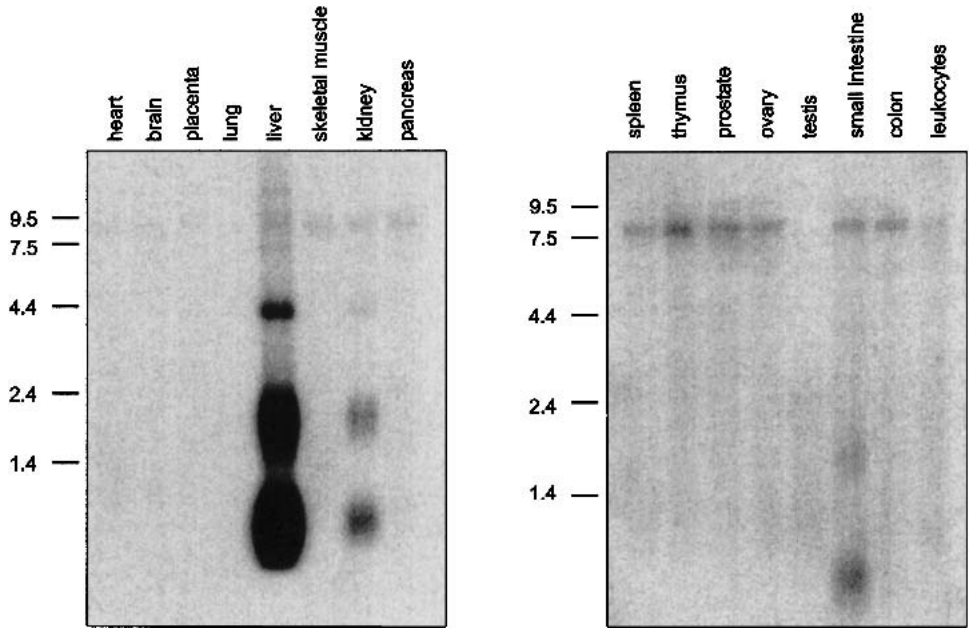

Figure 4. Northern Blot analysis. Human MTN Blots I+II (Clontech, A+B) were hybridized under high-stringency conditions with a ^{32}P-labeled LEAP-2-specific cDNA fragment. A transcript size of 0.7 kb identified in liver, kidney, and small intestine represents the complete LEAP-2 cDNA including a poly(A)-tail of ~200 bp. The signal at 2.0 kb corresponds to the LEAP-2 form coded by the distal promoter, which could also be identified in 5′-RACE-PCR, whereas the bands at 4.2 kb and 8.0 kb might either represent additional alternative promoter variants or homologous proteins related to LEAP-2.

parison of the successfully cloned partial murine LEAP-2 (*mLEAP-2*) gene with the murine cDNA concluded from the overlapping EST clones revealed that the murine gene also consists of three exons and two introns. The murine cDNA codes for a 76-residue protein, which shows a high sequence similarity (83%) to human LEAP-2. Most significantly, the putative mature murine LEAP-2 of 40 amino acid residues is entirely identical to human LEAP-2-(38–77). Using Northern analysis, we could identify liver and small intestine as main organs expressing *mLEAP-2*. LEAP-2-specific cDNA was also detected in tissue of pig, guinea pig, rhesus monkey, and of bovine origin (Fig. 5).

Expression analysis

To evaluate the expression pattern of the two main splice variants *LEAP-2*^{350} and *LEAP-2*^{550}, we performed a quantitative real-time RT-PCR analysis using primers suitable for the specific detection of either a completely spliced LEAP-2 cDNA or an intron 1-retaining variant. Liver, kid-

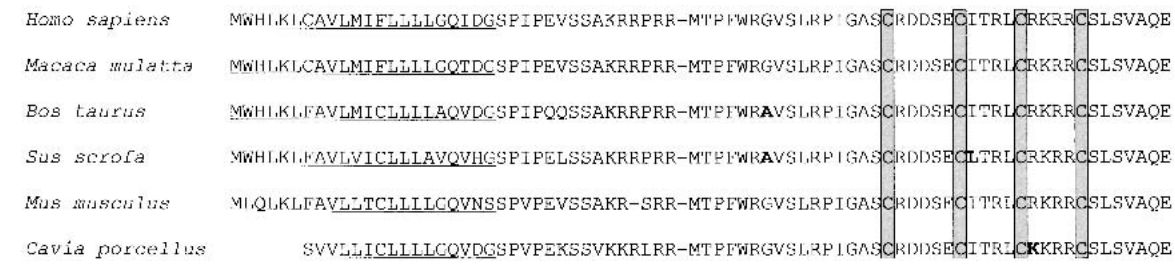

Figure 5. Alignment of LEAP-2 from mammalian species. Standard PCR carried out with primers originally designed for the human LEAP-2 gene revealed homologous LEAP-2 forms in rhesus monkey, cow, pig, mouse, and guinea pig. The depicted putative peptide sequences are deduced from the cDNA sequences obtained. Underlined amino acids represent the putative signal peptide sequences as predicted by the SignalP V2.0 program (Nielsen et al. 1997), and the mature LEAP-2 (38–77) form is hyphenated. The nucleotide sequence data reported in this paper have been submitted to the GenBank/EBI Data Bank with accession numbers AJ306405 (*Homo sapiens* mRNA), AJ409013 (*Sus scrofa* mRNA), AJ409014 (*Bos taurus* mRNA), AJ409054 (*Cavia porcellus* mRNA), AJ409055 (*Mus musculus* mRNA), AJ409056 (*Macaca mulatta* genomic DNA), AJ409063 (*Mus musculus* genomic DNA), AJ409064 (*Homo sapiens* genomic DNA), and AJ409065 (*Homo sapiens* alternative promoter sequence).

ney, antrum, duodenum, jejunum, bladder, and colon preferentially expressed *LEAP-2*^{350} rather than *LEAP-2*^{550}, ranging from an 80-fold excess of *LEAP-2*^{350} in bladder to a fourfold excess in colon (Fig. 6). The assay, however, did not discriminate if the splicing variants were derived from the distal or proximal promoter. Notwithstanding, we assume that the proportion of the completely spliced variant contributed by the distal promoter (*LEAP-2*^{915}; Fig. 3B) is negligible as its number is far less than the number of transcripts representing an intron 1-retaining form (*LEAP-2*^{1115}; Fig. 3B), whose expression was at the same time at least fourfold less (Fig. 6) than the transcripts of *LEAP-2*^{350}.

Chemical synthesis of LEAP-2

Synthetic LEAP-2-(38–77) and LEAP-2-(44–77) were assembled successfully using standard Fmoc chemistry. After we had determined the disulfide connectivity (Cys^{54}–Cys^{65} and Cys^{60}–Cys^{70}) of native LEAP-2 peptides, LEAP-2 was initially synthesized using a selective approach with pairwise trityl and acetamidomethyl protection for the cysteine residues, guaranteeing a selective formation of the two disulfide bonds required. The purified product obtained from this synthetic strategy eluted identically on analytical C18 HPLC, migrated identically in capillary zone electrophoresis, had an identical mass spectrum in ESIMS and was proved to exhibit the LEAP-2 primary structure by sequence analysis. In addition, limited proteolysis of synthetic LEAP-2 unambiguously revealed the presence of the disulfide bonding pattern that was determined for the native counterpart. Therefore, synthetic LEAP-2 is identical to the native peptide.

Interestingly, the investigation of the oxidative folding reaction of a LEAP-2 precursor with four thiol groups in alkaline milieu resulted in a dominating product that coeluted with the corresponding product obtained from the selective chemical approach described above. This indicates that linear LEAP-2 peptides contain sufficient information to generate a molecular form exhibiting a preferred three-dimensional structure stabilized by the two disulfide bonds.

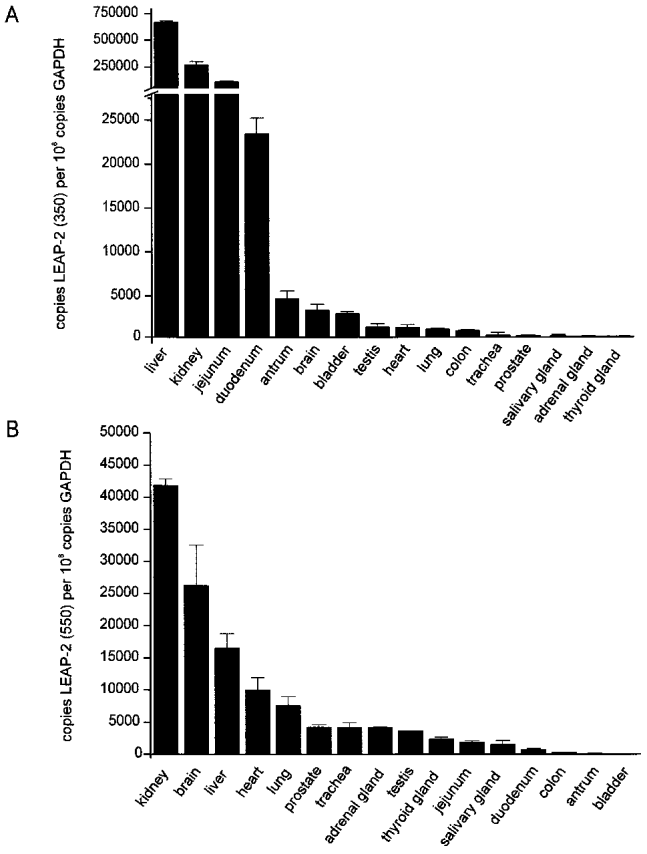

Figure 6. Tissue distribution of the two main LEAP-2 transcripts. Expression analysis of the LEAP-2 gene was performed by real-time quantitative RT-PCR specific for *LEAP-2*^{350} (*A*) and *LEAP-2*^{550} (*B*) transcripts. Standard curves were constructed using a reference plasmid representing a known number of copies of the target gene as well as the housekeeping gene. Expression levels are therefore depicted as number of copies of LEAP-2 transcript per 10^8 copies of the housekeeping gene GAPDH. Experiments were performed in duplicate at least twice.

Antimicrobial activity assays

LEAP-2 significantly shares structural characteristics (excess of positively charged amino acid residues, multiple disulfides) with antimicrobial peptides such as defensins and LEAP-1/hepcidin. We tested the antimicrobial activity using a zone inhibition assay monitoring the effects on the growth of microbes (Lehrer et al. 1991). In this assay, Gram-positive *Bacillus megaterium* ATCC14581 (83 inhibition units/IU ± 10.6 for 11 μg/well synthetic LEAP-2; mean ± S.D., $n = 3$), *Bacillus subtilis* ATCC6051 (72 IU ± 4.3), *Micrococcus luteus* ATCC9341 (20 IU ± 2.3), and *Staphylococcus carnosus* DSM20501 (55 IU ± 5.0), Gram-negative *Neisseria cinerea* ATCC14685 (49 IU ± 0.6), and yeasts *Saccharomyces cerevisiae* ATCC9763 (55 IU ± 5.0) (Fig. 7) and *Rhodotorula muciloginosa* DSM70403 (21 IU ± 8.0) were sensitive to treatment with synthetic LEAP-2-(38–77). The peptide did not affect the growth of Gram-negative *Escherichia coli* BL21 and *Pseudomonas fluorescens*. This antimicrobial activity of LEAP-2 was dose-dependent (Fig. 7). A product obtained from the treatment of LEAP-2 with the endoproteases subtilisin or Lys-C was completely inactive. Importantly, amino-terminally truncated LEAP-2-(44–77), which is another circulating and processed form, had no impact on the growth of the germs tested, regardless of whether we chose synthetic or native material. Therefore, the in vitro antimicrobial activity is dependent on the amino-terminal length of LEAP-2. We additionally carried out a dose-response colony-forming unit (CFU) assay to confirm these data. The half-maximal inhibitory activity (IC_{50}) of LEAP-2-(38–77)

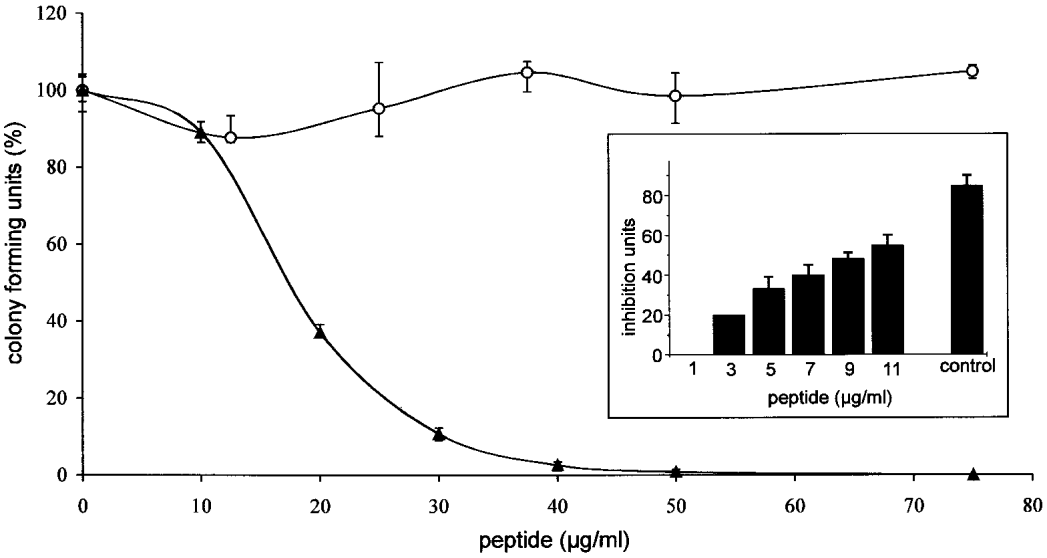

Figure 7. Antimicrobial activity of LEAP-2-(38–77) and LEAP-2-(44–77). Colony-forming unit assay of synthetic LEAP-2-(38–77) (solid triangles) and LEAP-2-(44–77) (open circles), the two main peptide forms isolated from hemofiltrate, against *S. cerevisiae* ATCC9763. Incubation without peptide represents 100% CFU. Synthetic and native LEAP-2-(44–77) led to similar results. The bars indicate the minimum and maximum value of the triplicates used in this representative assay. The inset depicts the dose-dependent effect of LEAP-2-(38–77) against *S. cerevisiae* in a radial diffusion assay. LEAP-2-(44–77) showed no effect in this sensitive antimicrobial assay. The antimicrobially active casocidin-I (11 µg/well) served as a positive control (Zucht et al. 1998). One inhibition unit (1 IU) corresponds to 0.1 mm diameter of growth inhibition zone, and the error bars represent the S.D. calculated from three experiments performed.

was ~5 µM against *S. cerevisiae* (Fig. 7) and *B. subtilis* (data not shown), corresponding to the activity of other antimicrobial peptides on a molar basis (Ganz 1999).

Discussion

We report here the isolation and characterization of LEAP-2, a new mammalian peptide secreted into the blood, which contains two disulfide bonds and exhibits antimicrobial activity in vitro. LEAP-2 mRNA codes for a precursor protein of 77 amino acid residues and is found predominantly in liver. Native LEAP-2 peptides of different length were isolated from human blood ultrafiltrate by HPLC and characterized by capillary zone electrophoresis, ESIMS, and amino acid sequence analysis. A LEAP-2 metabolite was also identified in urine of healthy donors. Similarities in primary structure and disulfide bonding between LEAP-2 and any other known protein have not been found.

The human LEAP-2 peptides isolated were 3–4.5 kD in size, and the longest form, LEAP-2-(38–77), which is most probably generated by a proprotein convertase from the precursor, has an overall charge of +4 at neutral pH. The predicted pI is 9.2, which is similar to other cationic peptides with in vitro antimicrobial activity. Whereas sequence regions exhibiting a significant hydrophobicity are not obvious from the LEAP-2 primary structure, the peptide contains a notable basic amino acid cluster -RKRR- (positions 66–69) typical for many antimicrobially active peptides (Andreu and Rivas 1998; Chen et al. 2000) as well as the highly acidic segment -DDSE- (positions 56–59). The spatial separation of acidic and basic amino acid residues along the peptide chain might allow interchain interaction of LEAP-2 molecules and contact with microbial cell membranes. No elements of secondary structure such as β-sheet and α-helix were detected by circular dichroism spectroscopy in aqueous solvents or in structure-promoting solvents containing trifluoroethanol. Notwithstanding, LEAP-2 may exhibit a defined tertiary structure stabilized by the disulfide bonds. Nuclear magnetic resonance studies are currently underway to address the question of whether LEAP-2 exhibits 3D-structural properties resembling other peptides. The characteristic structural features of LEAP-2 may have contributed to the difficulties we encountered in producing specific anti-LEAP-2 antibodies. Another significant characteristic of LEAP-2 is the lack of antimicrobial activity of the amino-terminally truncated LEAP-2-(44–77).

From the cDNA structure of LEAP-2, a precursor protein of 77 amino acid residues is deduced. Using the Signal P program (Nielsen et al. 1997), a 22-residue secretory signal peptide is predicted with a highly significant score. Cleavage between Gly22 and Ser23 would generate a 55-residue proprotein of LEAP-2. The isolated bioactive peptide is probably released from this proform via processing by a member of the furin family of proprotein convertases (PCs).

The highly basic region located amino-terminally to Met38 fulfills crucial requirements for a furin cleavage site (Nakayama 1997). The known abundance of PCs in liver tissue may account for the fact that no pro-LEAP-2 was identified in blood ultrafiltrate. In this respect, LEAP-2 closely resembles the recently identified LEAP-1/hepcidin, another multifunctional peptide from the liver (Krause et al. 2000; Park et al. 2001). The occurrence of smaller LEAP-2 peptides in blood indicates a further proteolytic processing.

Based on our experimentally determined peptide sequence, no homologs with regard to the primary structure and the cysteine pattern were found in protein databases. Screening EST databases resulted in related cDNA homologs from pig and mouse, and LEAP-2-related PCR products were obtained by PCR performed with cDNA of guinea pig, rhesus monkey, and bovine origin (Fig. 5). The corresponding translated cDNAs revealed an extremely high degree of amino acid conservation among these species, in particular with regard to LEAP-2-(38–77), the putatively mature peptide produced by a furin-like enzyme. Moreover, the putative endoproteinase cleavage sites are completely conserved (Arg–Arg recognition sequence).

The identification of several alternative splicing products of *LEAP-2* as well as an mRNA isoform containing an extended 5′-region reflects well the recent findings of the human genome sequencing effort, indicating that one gene frequently gives rise to more than a single transcript or protein. Alternative promoter usage, which is often combined with alternative splicing events, provides additional flexibility in the control of gene expression, including temporal or tissue-specific gene expression and translational efficiency of the mRNA (Ayoubi and van de Ven 1996). In the case of *LEAP-2*, we observed a tissue-specific distribution of different splicing variants (Fig. 3) and the mRNA isoform most probably transcribed to the isolated peptide is coded mainly by the proximal promoter. Alternative promoter usage is involved in a variety of regulatory mechanisms (Kozak 1991, 1996), and the functionality of transcripts derived from the distal LEAP-2 gene promoter remains to be evaluated. For instance, the incorporation of intron 1 into an open reading frame would give rise to a peptide MSLPLQIDGSPIPEVSSAKRRPRR-LEAP-2-(38–77) (see accession number AJ409064), a precursor that would still contain a putative furin cleavage site, but the lack of a signal peptide sequence would impede secretion of the peptide. On the other hand, the occurrence of incompletely spliced transcripts, which are posttranscriptionally processed on stimulation with mitogen activators, has been reported (Kozak 1996).

In conclusion, we have identified a novel peptide that is synthesized in the liver, secreted into the blood, subsequently processed and, at least partially, eliminated by the kidney. LEAP-2 is highly conserved among mammals and its primary structure and disulfide bond pattern exhibit no similarity to any known peptide. The expression appears to be well regulated at the transcriptional level. The putatively mature LEAP-2-(38–77) is antimicrobially active in vitro at concentrations in the micromolar range, a potency comparable with other antimicrobial peptides. Further studies, in particular regarding the identification of genes regulated by LEAP-2 and the induction of cytoplasmic signaling cascades, are required to determine the physiological role of LEAP-2.

Materials and methods

Identification of LEAP-2 from blood ultrafiltrate

LEAP-2 was identified during a comprehensive search for bioactive peptides based on a peptide library generated from human blood ultrafiltrate using an approach that we described previously in general and for the isolation of other novel biologically active peptides (Schulz-Knappe et al. 1997; Mägert et al. 1999; Detheux et al. 2000). Briefly, hemofiltrate obtained from patients with chronic renal failure was collected at the Nephrologisches Zentrum Niedersachsen (Hannoversch-Münden, Germany) in quantities of 1600–2000 L/week. The sterile and acidified hemofiltrate was loaded onto a strong cation exchanger. Stepwise elution of the peptide material was achieved with subsequent washes using seven buffers with increasing pH. Each of the seven pools obtained was then subjected to reversed-phase HPLC, resulting in a total of about 250–300 peptide subfractions derived from human hemofiltrate.

Starting with fraction 12 of pool 6, LEAP-2 peptides were isolated and characterized after four subsequent steps of chromatography: (1) cation exchange HPLC (Parcosil Prokat, Biotek, Östringen, Germany; 125 × 20 mm, 7 μm, 300 Å; flow, 10 mL/min; eluent A, 25 mM sodium acetate, 25 mM acetic acid, pH 4.5; eluent B, 1 M NaCl, 25 mM sodium acetate, 25 mM acetic acid, pH 4.5; linear gradient, 0%–100% (vol/vol) B in 42 min); (2) analytical reversed-phase HPLC (Vydac C18, The Separations Group, Hesperia, CA; 250 × 4.6 mm, 5 μm, 300 Å; flow, 0.7 mL/min; eluent A, 0.1% (vol/vol) TFA in water; eluent B, 0.1% TFA in acetonitrile; linear gradient, 0%–60% B in 55 min); (3) analytical cation exchange HPLC (Parcosil Pepkat, Biotek; 50 × 4 mm, 5 μm, 300 Å; flow, 1 mL/min; eluent A, 25 mM phosphate, pH 3.0; eluent B, 1 M NaCl, 25 mM phosphate, pH 3.0; linear gradient, 0%–60% B in 60 min); and (4) desalting by reversed-phase HPLC (Parcosil C4, Biotek; 50 × 4 mm, 300 Å; flow 0.7 mL/min; eluent A, 0.1% TFA in water; eluent B, 0.1% TFA in acetonitrile). The fractions were analyzed by capillary zone electrophoresis (P/ACE 2000, Beckman, Munich, Germany; buffer, 0.1 M NaH_2PO_4 containing 0.2 mg/mL hydroxypropylmethylcellulose, pH 2.5; uncoated capillary, 50 cm × 50 μm, pressure injection; constant current, 50 μA; UV detection at 200 nm, 25°C) and electrospray mass spectrometry (ESIMS, Sciex API III, Perkin Elmer, Langen, Germany). Fractions containing pure LEAP-2 peptides were lyophilized and subjected to amino acid sequence analysis.

Structural characterization of LEAP-2

The primary structure of purified LEAP-2 peptides was determined by automated Edman degradation on a Procise 494 sequencer (Applied Biosystems, Foster City, CA). Prior to sequence

analysis, the peptides were treated with excess dithiothreitol to reduce disulfide bonds. To determine the disulfide pattern of native LEAP-2, the peptide was subjected to proteolytic digestion with trypsin at ambient temperature for 24 h without dithiothreitol treatment. The fragments obtained were separated by HPLC (Nucleosil C18 column, Macherey & Nagel, Düren, Germany) and characterized by ESIMS and amino acid sequencing.

Chemical synthesis of LEAP-2 and disulfide formation

Synthetic LEAP-2 corresponding to the amino acid sequence of LEAP-2-(38–77) was assembled using standard Fmoc (fluorenylmethoxycarbonyl) chemistry on a preloaded TentaGel S PHB resin (Rapp Polymere, Tübingen, Germany) on a 433A peptide synthesizer (Applied Biosystems) at a scale of 0.1 mmole. Activation of Fmoc-protected amino acids was carried out with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). Cysteine residues were trityl-protected. For cleavage and deprotection, the dry peptidyl resin was treated with trifluoroacetic acid/ethanedithiol/water 94:3:3 (vol/vol/vol) for 3 h. The product was then precipitated by adding cold tert-butylmethylether. The crude material was dried, dissolved in water, filtered and prepurified by preparative Vydac C18 HPLC. The combined fractions containing the reduced product were subjected to oxidative folding at a concentration of 0.5 mg/mL in 0.1 M NH_4HCO_3 (pH 8.3) at room temperature for 48 h. Only a minor amount of polymeric material has been detected during this refolding step. The chromatographically homogeneous LEAP-2 obtained from this mixture by preparative C18 HPLC was lyophilized, and the molecular mass determined by ESIMS was 4581.3 D (calculated 4581.3 D). Alternatively, LEAP-2-(38–77) and LEAP-2-(44–77) were synthesized using an orthogonal protection scheme for the cysteine residues allowing selective introduction of the disulfide bonds. In this approach, Cys^{54} and Cys^{65} were trityl-protected, and Cys^{60} and Cys^{70} were acetamidomethyl-protected. Whereas the disulfide bridge between Cys^{54} and Cys^{65} was introduced by air oxidation, the disulfide bond connecting Cys^{60} with Cys^{70} was established by iodine treatment of the monocyclic precursors. The corresponding products obtained from both synthetic pathways and the isolated LEAP-2 were identical during capillary zone electrophoretic and ESIMS analysis, and exhibited identical cysteine connectivity. The overall yield of both synthetic approaches was in the range of 5%–8%.

Antimicrobial assay

A conventional inhibition zone assay was performed as described previously (Krause et al. 2000). Briefly, ~10^6 logarithmic-growth-phase germ cells were added to 100 mL of 10 mM sodium phosphate buffer (pH 7.2, 37°C) containing 0.02% (wt/vol) Tween 20, 0.3 mg/mL tryptic soy broth (TSB), 0.2% (wt/vol) type 1 agarose (low EEO, Sigma-Aldrich, Steinheim, Germany), and 0.6% (wt/vol) NuSieve GTG agarose (FMC BioProducts, Rockland, ME). After pouring this preparation into sterile Petri dishes, gel cylinders of 3 mm diameter were punched and filled with increasing dilutions of synthetic LEAP-2-(38–77) or LEAP-2-(44–77) (1–11 μg/11μL). After 18 h of incubation at 37°C, clear circular areas surrounding the cavities indicated the suppression of bacterial growth. Inhibition units (IU) were determined as described previously (Lehrer et al. 1991). Experiments were carried out in triplicate.

CFU assay

S. cerevisiae was cultured at 30°C in YPD medium (1% yeast extract, 2% peptone, 2% dextrose (wt/vol), pH 6.5). Cells were harvested by centrifugation at 600*g* and 4°C for 10 min when reaching an OD_{620} of 0.8. After washing the pellet, it was dissolved in 10 mM sodium phosphate buffer including 1% TSB (wt/vol). Inocula of ~2×10^5 colony-forming units (CFU) diluted in 100 μL medium were added to 1–7.5 μg of LEAP-2-(38–77) and incubated for 2 h at 30°C. The reaction was stopped by a 500-fold dilution with ice-cold 10 mM sodium phosphate buffer, and aliquots were spread manually on YPD agar plates. Following an overnight incubation at 30°C, colonies were counted and inhibition percentiles were calculated. An analogous procedure was performed with *B. subtilis* using LB medium (pH 7.4) at 37°C. A minimum of three separate experiments was performed, each in triplicate.

Molecular biological standard methods and cDNA cloning

RNA extraction, cDNA first strand synthesis, PCR, RT-PCR, and chromosomal localization of the LEAP-2 gene by radiation hybrid mapping were carried out as described previously (Mägert et al. 1998; Krause et al. 2000; Motzkus et al. 2000). Nested 5′-RACE-PCR was performed to clone the complete LEAP-2 cDNA sequence. Gene-specific primers (R-in, R-out; Fig. 2) were deduced from EST clones (GenBank accession numbers T60528, R93776), and sequence extension was conducted with Marathon-Ready human liver and lung cDNA (Clontech, Palo Alto, CA). All bands obtained were characterized by DNA sequencing. Standard PCR primers (E1-S, E3-AS) derived from 5′-RACE-PCR products were suitable to isolate *LEAP-2^{350}*-specific cDNAs from six different species using an annealing temperature of 60°C. The amplicons isolated from liver (human, mouse, bovine), small intestine (pig, guinea pig), and rhesus monkey genomic DNA (Clontech) were cloned into pGEM-T vector (Promega, Mannheim, Germany). For determination of nucleotide sequences, at least four independent clones were sequenced on a Prism 310 Genetic Analyzer (Applied Biosystems). To demonstrate that *LEAP-2^{480}* was a PCR artefact (Fig. 3A), we used clones containing *LEAP-2^{350}* and *LEAP-2^{550}* and reisolated the inserts by digestion with 2 U of Sst-I and Sst-II (Life Technologies, Karlsruhe, Germany) for 16 h at 37°C. After pooling the two reisolated cDNAs, subsequent separation on an agarose gel led to two bands of the expected size, whereas three bands (350 bp, 480 bp, and 550 bp) were detected when the pooled cDNAs were treated for 5 min at an annealing temperature of 60°C prior to electrophoresis.

Northern blot analysis

Northern blots with preloaded poly(A^+) RNA from 16 normal human tissues were purchased from Clontech (Human MTN Blot I+II) and performed according to the manufacturer's instructions. A 304-bp cDNA-fragment obtained with the primers MTN-S (5′-CAACATCCTCCCCCTGTCAAGATG-3′) and MTN-AS (5′-ggagcattgtcggaggtgac-3′) served as hybridization probe. cDNA was labeled with [α-^{32}P]-CTP using a random primed DNA-labeling kit (Roche Diagnostics, Mannheim, Germany). Hybridization was carried out for 1 h at 68°C and final washes were performed under high-stringency conditions.

Real-time quantitative RT-PCR

Real-time quantitative RT-PCR analysis was carried out using a Prism 7700 Sequence Detection System (Applied Biosystems).

The theoretical background for this highly sensitive and accurate method of cDNA quantification has been described elsewhere (Bièche et al. 1998). The amplification system for the human 18S-rRNA gene and the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, which served as housekeeping genes to ensure consistent loading of cDNA and PCR efficiency, comprised primers generating one 74-bp PCR product each and the fluorescent probe 18S-P, 5′-(FAM)-TCAGTTATGGTTCCTTTGGTC GCTCGC-(TAMRA)-3′ or GAPDH-P, 5′-(FAM)-cctggccaaggt catccatgacaac-(TAMRA)-3′, respectively. The two main transcripts of LEAP-2, *LEAP-2*^{350}, and *LEAP-2*^{550} (Fig. 3A), were amplified by utilizing the same probe and reversed primer, but a different forward primer. Whereas the probe LEAP-2 P 5′-(FAM)-CCCAATACCAGAAGTGAGTTCGGCAAAG-(TAMRA)-3′ hybridized to exon 2, the reversed primer LEAP-2 RV (5′-GAAC AGCGTCTTTTTCTGCATAG-3′) flanked intron 2. We used *LEAP-2*^{350} F (5′-GCTGTTGGGCCAGATAGATG-3′) as specific forward primer for *LEAP-2*^{350} amplification and *LEAP-2*^{550} F (5′-GTCTTTGCCCTTACAGATAGATGG-3′) for *LEAP-2*^{550}. Amplification mixes (25 μL) were prepared with TaqMan Universal Master Mix (Applied Biosystems). Concentration of primers and probe was 250 nM and 200 nM, respectively. The corresponding amplification products were verified as gene-specific by DNA sequencing. Total RNA from jejunum, duodenum, antrum, and urinary bladder was extracted from normal tissue provided by an organ donor program, whereas all other total RNAs were purchased from Clontech. A standard curve was constructed with a reference plasmid that contained the particular PCR product. After quantifying the PCR products spectrophotometrically, sixfold serial dilutions of the plasmid representing 10^5 to 10 copies of the target amplicon were generated. The number of copies in each sample was automatically calculated by the Prism 7700 system according to the standard curve, and finally the number of copies per sample was normalized to the number of copies of 18S rRNA or GAPDH.

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