Molecular Cloning and Characterization of a Novel Mammalian Endo-apyrase (LALP1)*

Received for publication, December 21, 2000, and in revised form, February 12, 2001
Published, JBC Papers in Press, February 23, 2001, DOI 10.1074/jbc.M011569200

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Here we describe the cloning, localization, and characterization of a novel mammalian endo-apyrase (LALP1) in human and mouse. The predicted human LALP1 gene encodes a 604-amino acid protein, whereas the mouse Lalp1 gene encodes a 606-amino acid protein. The human and mouse genes have 88% amino acid sequence identity. These genes share considerable homologies with hLALP70, a recently discovered mammalian lysosomal endo-apyrase. The human LALP1 gene resides on chromosome 10q23–q24 and contains 12 exons and 11 introns covering a genomic region of ~46 kilobase pairs. The subcellular localization and enzymatic activity of LALP1 indicated that LALP1 is indeed an endo-apyrase with substrate preference for nucleoside triphosphates UTP, GTP, and CTP.

Apyrases are hydrolytic enzymes that cleave nucleoside tri- and diphosphates in a calcium- or magnesium-dependent manner but are insensitive to P-, F-, or V-type ATPase inhibitors (1). Both apyrases and ATPases can use ATP as substrates; however, there are substantial differences (1, 2). For instance, the ATPases are highly selective toward their substrate ATP with ADP and phosphate as reaction products. On the contrary, apyrases can use different nucleoside tri- and diphosphates as their substrates, with phosphates and nucleoside monophosphates as the main reaction products. Additionally, sequence comparison among various apyrases revealed the presence of apyrase conserved regions, which are highly conserved among all apyrases from various organisms such as plants, parasites, and mammals. Thus far, two types of apyrases, ecto-apyrases and endo-apyrases, have been described (2). Ecto-apyrases such as CD39 are apyrase enzymes with their catalytic domain exposed on the cell surface (1–3) and are believed to be involved in many processes such as neurotransmission (4), platelet aggregation, and blood pressure regulation (5). On the other hand, endo-apyrases such as uridine diphosphatase and the 700-kDa lysosomal apyrase-like protein (LALP70) are apyrase enzymes with their catalytic domain localized intracellularly (6, 7). The biological functions of the endo-apyrases are unknown, although it was suggested that they are important for regulating the level of activated sugar during protein glycosylation.

Recently, Wang and Guidotti (6) identified the first mammalian endo-apyrase, human uridine diphosphatase. This enzyme is predicted to be a 610-amino acid protein with two putative transmembrane domains. Using a myc-tagged version of this protein, this enzyme was found to be in the luminal side of the Golgi apparatus and had the highest catalytic activity using UDP as its substrate and had lower activities using GDP, CDP, UTP, GTP, CTP, and TTP. Interestingly, Biederbick et al. (7) reported altered substrate preferences of LALP70, which is a splicing variant of uridine diphosphatase and has an additional 8 amino acids (VSFASSQQ) resulting from the inclusion of an alternate exon. The enzymatic properties of this splice variant revealed a broad substrate specificity, with CTP, UTP, GDP, CTP, and GDP as preferred substrates. Using antibodies and a green fluorescent protein-tagged version of this splicing variant, LALP70 was co-localized with the autophagic marker monodansylcadaverine and the lysosomal protein lamp1, suggesting a lysosomal/autophagic vacuole subcellular location (8). Although most of the transiently expressed LALP70/GFP1 fusion protein was co-localized with lamp1-positive vacuoles, an association with the Golgi apparatus and the endoplasmic reticulum could not be ruled out. These studies clearly demonstrated that LALP70/uridine diphosphatase is the first mammalian member of the endo-apyrase gene family. Here we report the molecular cloning and characterization of the LALP1 gene, which closely resembles LALP70 in both structure and enzymatic property. Hence, we propose that LALP1 is a second member of the endo-apyrase gene family.

**EXPERIMENTAL PROCEDURES**

5′-RACE and 3′-RACE—5′- and 3′-RACE reactions were conducted with the SMART RACE kit (CLONTECH) according to the manufacturer's instructions, with a slight modification as described previously (11, 12). Briefly, for 5′-RACE, the first-strand cDNA synthesis is primed using a gene-specific primer and a SMART oligonucleotide with human brain total RNA. After reverse transcription reaches the end of the mRNA template, several dC residues are added to the end of the cDNA. The SMART oligonucleotide, which contains several 3′dG at its 3′end, anneals to the tail of the newly synthesized cDNA and then serves as a template for further extension of the cDNA by RT. After the RT reaction, an internal gene-specific reverse primer and a UP primer, which is complementary to SMART oligonucleotide, were used to perform PCR using the RT products as templates. To increase the specificity and

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* This research was supported in part by National Institutes of Health Grants 1R01DK53266-01 (to J.-X. S) and AG015688 (to D. H. W). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ Supported by a University of Florida Alumni Fellowship.

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1 The abbreviations used are: GFP, green fluorescent protein; RACE, rapid amplification of cDNA ends; RT, reverse transcription; PCR, polymerase chain reaction; bp, base pair(s); UTR, untranslated region.
product yield of 5′-RACE, nested PCR was then performed using an
other internal gene-specific primer and NUP primer (internal primer of
UP primer). For 3′-RACE, the first-strand cDNA was synthesized using
a modified oligo(dT) with a UP oligonucleotide tail.

The UP primer and a gene-specific forward primer were used for
first-round PCR. Nested PCR was performed using NUP and an internal
gene-specific forward primer. PCRs were carried out in a final
volume of 20 µL. After the RT-PCR, the samples were denatured at
94 °C for 5 min; amplifications were carried out with 5 cycles of 30-s
denaturing at 94 °C, 30-s annealing at 94 °C, 30-s annealing at 62 °C, and a 4-min extension at
94 °C for 5 min; amplifications were carried out with 5 cycles of 30-s
Denaturing at 94 °C, 30-s annealing at 68 °C, and a 4-min extension at
72 °C followed by 30 cycles of 30-s denaturing at 94 °C, 30-s annealing at 62 °C, and a 4-min extension at 72 °C. PCR products obtained from
nested PCR were loaded onto a 2% agarose gel, and individual bands
were excised from the gel for direct sequencing. The PCR product was
subcloned into TA vector (Invitrogen, San Diego, CA), and the sequence was
determined with ABI 310 DNA sequencer as described previously (12–14).

Expression Analysis by Competitive RT-PCR—Total RNA was isolated
from various mouse tissues using the RNeasy kit (Qiagen) according to
instructions. Transfected cells were harvested for the apyrase assay
determination by transfection experiments with the LALP1-
expressed pEGFP fusion protein.

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**TABLE I**

| Primer name | 5′–3′ Sequence |
|-------------|----------------|
| Human       |                |
| hLALP1-RT   | ATACAAAGGAGAACGAGGACAC |
| hLALP1-R1   | GCTCGTGTGGTCGGTGGTGG |
| hLALP1-R2   | CTCGATGTTGAAATCTG |
| hLALP1-F1   | ACTTTCTACGGTAAATCTCTT |
| hLALP1-F2   | CAGCCCCTTCTTCCTTC |
| hExon8-F    | ACAGCGGATGAGGGTTT |
| hExon8-R    | AGGCGTCCCGGGCAGATTTG |
| h exon1-F   | ATGGAGATGCTGGGACAG |
| Mouse       |                |
| m exon5F    | CCAAATCACCTCAGACAGGA |
| 5UTR-R      | GGCGCAACAAGAAGAATCAAGG |
| mLALP1-R    | CTTGCCAGAGATACATCTT |
| mLALP1-F1   | GGCACAGTGAGTACCAGGGA |
| mLALP1-F2   | AAAACACGATTTCTACATC |
| β-actin-F   | CTCGGCTTCCCTTTGTCTAC |
| β-actin-R   | GAAAAGATGACCCAGATCATG |
| Primers for RACE |         |
| Smart oligonucleotide | AAGCAGCTGTATCAACCCAGACATG |
| UP          | CTATAACAGCTACATATGAAGCAACGACAG |
| NUP         | AAGCAGCTGTATCAACCCAGACAG |

**RESULTS AND DISCUSSION**

**Cloning of the Human LALP1 Gene**—The human LALP1 gene
was cloned using SMART RACE technology. Based on the
sequence of expressed sequence tag N35618 in the human chromo-
somal region 10q23–q24 (9, 10), several gene-specific reverse
primers (hLALP1-RT, hLALP1-R1, and hLALP1-R2) and forward
primers (h LALP1-F1 and hLALP1-F2) were designed for
5′-RACE and 3′-RACE, respectively. The resulting full-length
sequence (Fig. 1) was 2962 bp (GenBank™ accession number
AF269255). A GenBank™ search indicated that the human
LALP1 gene is contained in clone RP11-483F11 (GenBank™
average accession number AL133353). Comparison of cDNA and genomic
sequences suggested that LALP1 has 12 exons that expand about
46 kilobase pairs of genomic sequence (Fig. 2). The cloned cDNA
coincides almost exactly with the computer-predicted structure. The
translation initiation codon ATG is located near the end of
exon 1, which contains only eight coding nucleotides. Multiple
in-frame stop codons are present upstream of the putative first ATG. A CpG island was identified at 2124 bp upstream of the putative first ATG. A putative polyadenylation signal sequence (AATAAA) is found at 28 bp upstream of the poly(A) tail. The LALP1 gene contains 1,812 nucleotides of coding sequence and encodes a protein of 604 amino acids with a molecular mass of 69.88 kDa. Interestingly, the exon/intron structure of LALP1 is almost identical to that of the LALP70 gene (7).

Cloning of the Mouse Lalp1 Gene—The mouse Lalp1 gene was cloned based on homology with its human homologue. Briefly, two human LALP1 primers (hExon4-F and hExon8-R) were used to amplify the mouse gene from mouse brain cDNA. A fragment of 450 bp encompassing the region from exon 4 to exon 8 was obtained and directly sequenced. This mouse fragment shares 86.8% homology with the human LALP1 homologous region, confirming that the fragment is indeed derived from the mouse Lalp1 gene. A mouse reverse primer (mLALP-R1) was designed based on the partial mouse Lalp1 sequence and used together with a human forward primer (hExon1-F) in RT-PCR. This RT-PCR generated a fragment of 730 bp, which shares 89.5% homology with the human LALP1 sequence. To obtain the complete DNA, RT-PCR was conducted using total RNA from various mouse tissues and the mExon8-5UTR-R primer pair (752 bp). A 450-bp β-actin fragment was co-amplified in the same PCR. PCR products were electrophoresed on a 2% agarose gel. The ratio of Lalp1/β-actin reflects the relative expression level of the Lalp1 gene.

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FIG. 1. Nucleotide and protein sequences of the human LALP1 gene. The four highly conserved apyrase regions (ACR1–4) are in italic boldface type. The putative membrane-spanning regions determined using the algorithm of Kyte and Doolittle (15) are underlined.

FIG. 2. Genomic structure of the human LALP1 gene. Black boxes represent exons, whereas the lines represent introns.

FIG. 3. Tissue distribution of Lalp1 in mice. RT-PCR was conducted using total RNA from various mouse tissues and the mLALP-R1 primer pair (752 bp). The mouse fragment was co-amplified in the same PCR. PCR products were electrophoresed on a 2% agarose gel. The ratio of Lalp1/β-actin reflects the relative expression level of the Lalp1 gene.
sequences at the 5' and 3' ends of the gene, 5'- and 3'-RACE were performed using several reverse and forward primers (mLALP1-RT, mLALP1-R2, mLALP1-F1, and mLALP1-F2).

Mouse Lalp1 cDNA contains 3268 bp (GenBank™ accession number AF288221) with an open reading frame of 1818 bp and encodes a protein of 606 amino acids (with a molecular mass of 69.99 kDa). The mouse and human cDNA sequences share 87% nucleotide sequence similarity in the entire coding region, whereas the sequence homology in 5'-UTR and 3'-UTR is poor, further supporting the open reading frame assignment. The deduced protein sequences are also highly conserved between human and mouse (88% identity).

To compare the relative expression levels in different tissues, competitive RT-PCR was performed with RNA from different mouse tissues. As shown in Fig. 3, Lalpl is highly expressed in most tissues including the brain, kidney, liver, and testis. The expression is relatively lower in the lung, thymus, and heart. Among all tissues analyzed, the expression is lowest in the spleen. These results suggest that the LALP1 protein must play critical roles in the cellular functions of many different tissues.

Nucleotide Phosphatase Activities—A BLAST search revealed that the LALP1 and Lalpl proteins have significant sequence homologies with human LALP70 and guanosine diphosphatase protein family members (Fig. 4). The human LALP1 shares 59% identity and 71% similarity to LALP70, 58% identity and 70% similarity to guanosine diphosphatase, and 25% identity and 42% similarity to CD39. These results suggest that LALP1 may be an apyrase.

To determine whether LALP1 is indeed an apyrase, nucleotide phosphatase activity was measured in HEK293 cells transfected
with the pEGFP vector or the full-length LALP1 cDNA constructs (pEGFP/LALP1). The experiments were done in the presence of 1 mM azide (inhibitor of F-type ATPase) and 0.5 mM vanadate (inhibitor of P-type ATPase), which do not inhibit the activities of E-ATPases (1–2). As shown in Fig. 5, the mean activities were higher for cells containing LALP1 expression vector than in cells containing control vector. For example, the CTPase activity (24.9 ± 1.46 nmol P_i/min/mg) with the LALP1 construct is 5-fold higher than the CTPase activity (4.34 ± 2.02 nmol P_i/min/mg) for cells transfected with control vector. Student’s t test suggested that the mean activities were significantly different between LALP1 and control vector with UTP (p < 0.002), GTP (p < 0.002), and CTP (p < 0.0001) as the substrates (Fig. 5). Furthermore, the nucleotide phosphatase activities are absolutely dependent on the presence of Ca^{2+} ions (data not shown). Therefore, our data indicate that human LALP1 is truly an appyrase with a substrate preference for UTP, GTP, and CTP. It is interesting to note that the HEK293 cells have very high enzymatic activities for each of the eight nucleotides and each of the two cell lines were assayed in three independent experiments. The mean and S.D. of the three experiments are presented.

In conclusion, we have cloned the human LALP1 and mouse Lalp1 genes that share a high degree of sequence homology with mammalian endo-apyrases. Studies of the enzymatic activities and intracellular localization strongly suggest that these genes encode a novel member of the mammalian endo-apyrase gene family.

Acknowledgments—We thank James Yang for performing statistical analyses and Drs. Predeep G. Kumar and Malini Laloraya for helpful discussion.

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J. Biol. Chem. 2001, 276:17474-17478.
doi: 10.1074/jbc.M011569200 originally published online February 23, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M011569200

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