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SHORT COMMUNICATION

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A human cDNA coding for the Leydig insulin-like peptide (Ley I-L)

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Abstract cDNA clones for the human Leydig insulin-like peptide (Ley I-L) have been isolated and characterized. The nucleotide sequence of the 743-bp cDNA includes an incomplete 7-bp 5′-noncoding region, an open reading frame of 393 bp, and a 343-bp 3′-noncoding region. By primer extension analysis, the transcription start site was determined as being 14-bp upstream of the translation start site. The underlying gene is expressed in the testis but not in other organs. From the cDNA sequence, it can be deduced that the Ley I-L protein is synthesized as a 131-amino-acid (aa) preproprotein and that it contains a 24-aa signal peptide. Comparison of the pro Ley I-L protein with members of the insulin-like hormone superfamily predicts that the biologically active hormone, after proteolytic processing of the C peptide, consists of a 31-aa long B chain and a 26-aa long A chain, and that it has a molecular weight of 6.25 kDa.

Introduction

Several established, albeit less well characterized, peptide growth factors have been localized to the testis and found to be produced by different cell types at different phases of testicular development (Bellvé and Zheng 1989; Söder et al. 1989). The physiological role of many of these growth factors in the testis is still unknown.

We recently described a porcine cDNA encoding a Leydig insulin-like peptide (Ley I-L) that is structurally related to members of the insulin-like superfamily (Adham et al. 1993). This family comprises insulin, relaxin, and insulin-like growth factors I and II. Some of these factors have been found to play a crucial role in spermatogenesis (Skinner 1991; Söder et al. 1992). The porcine Ley I-L gene is only expressed in prenatal and postnatal Leydig cells, and the protein is synthesized as a preproprotein. The mature hormone is similar to insulin in sequence and is composed of B and A chains that are connected by two disulfide bridges. We report here the isolation of the human Ley I-L cDNA and its specific expression in human testis.

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Materials and methods

Isolation and sequencing of human Ley I-L cDNA clones

Some 4×10^5 recombinants of a human testis cDNA library in λgt11 (Clontech, Palo Alto, USA) were screened using the plaque hybridization method (Benton and Davis 1977). The 450-bp porcine Ley I-L1 cDNA fragment (Adham et al. 1993) was labeled with [α-^{32}P]dCTP by the random hexanucleotide primer method (Feinberg and Vogelstein 1983). Hybridization was carried out at 60° C overnight in the following solution: 5 × buffer A (1 × buffer A consists of 10 m*M* NaH_2PO_4, pH 7.0, 180 m*M* NaCl, 1 m*M* EDTA), 5 × Denhardt's solution (1 × Denhardt's solution = 0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.02% Ficoll), 0.1% SDS, and salmon sperm DNA at 200 μg/ml. The filters were washed twice at 60° C to a final stringency of 0.2 × buffer A. Three cDNA clones that hybridized strongly with the porcine Ley I-L probe were isolated and characterized. Sequencing of the cDNA was performed by the double-stranded dideoxy chain termination technique (Sanger et al. 1977) using [α^{35}S] dATP (Amersham-Buchler, Braunschweig) and the Sequenase sequencing kit (US Biochemical, Cleveland, USA). The cDNAs were sequenced in both directions using vector-derived primers and synthetic oligonucleotides derived from the cDNA sequence. Sequence data were assembled and analyzed with the help of the DNA STAR computer program.

Primer extension analysis

Total cellular RNA was extracted from human testes by the guanidinium isothiocyanate method (Chirgwin et al. 1979), and poly(A)-rich RNA was prepared by oligo (dT) cellulose column chromatography (Aviv and Leder 1972). Synthetic oligonucleotides complementary to positions 43–63 of the human cDNA were 5′-endlabeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Sambrook et al. 1989). Approximately 5 ng labeld oligonucleotide was hybridized with 5 μg poly (A)-rich RNA and extended with cloned M-MLV reverse transcriptase (BRL) according to Domenjoud et al. (1990).

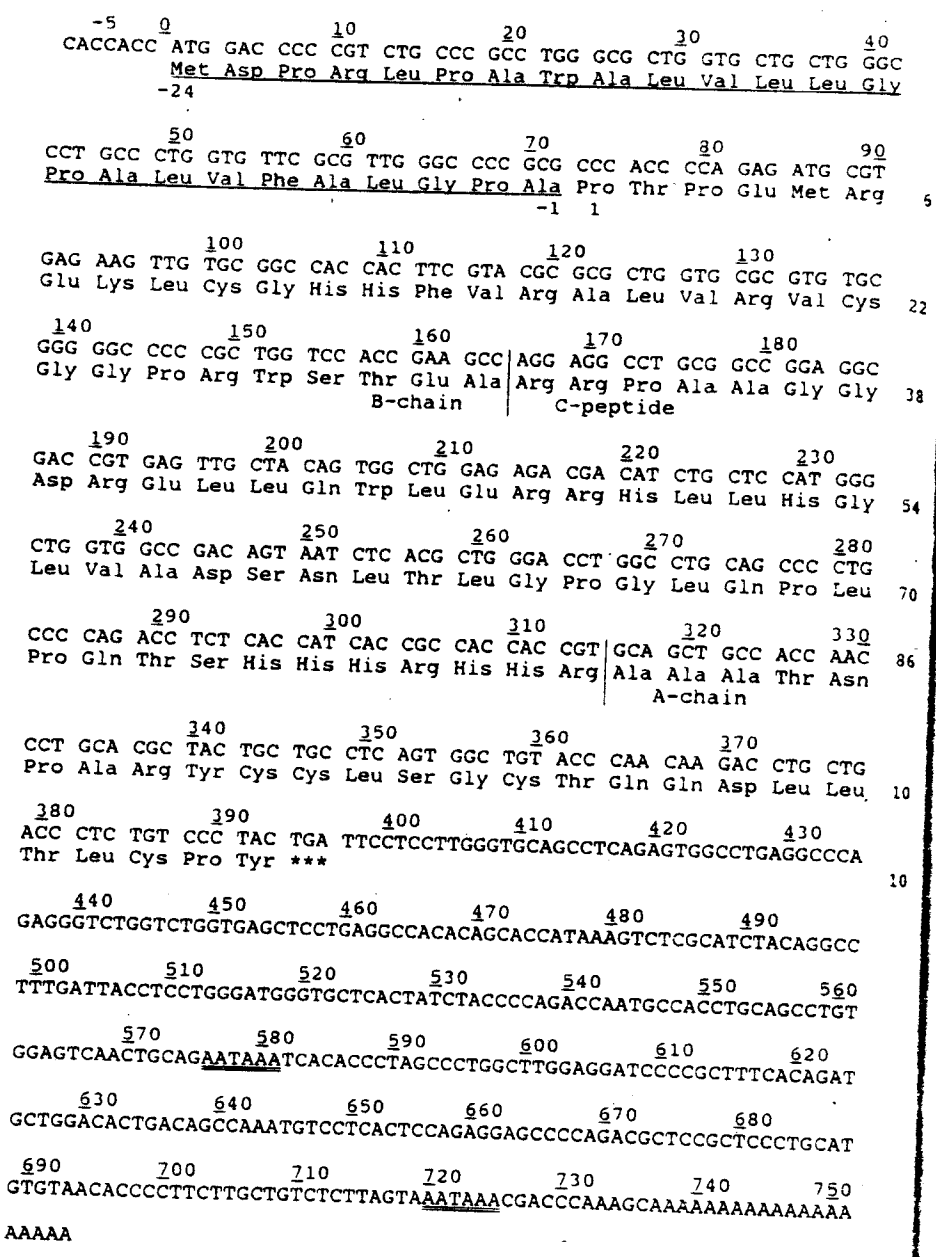

Fig. 1 Nucleotide and deduced amino acid sequence of human prepro Leydig insulin-like cDNA. *Numbering* of nucleotides is given *above* the cDNA sequence, whereas that of the amino acid sequence is given *right*. The signal peptide (–24 to –1) is *underlined*. The termination codon TGA is marked by *asterisks* and potential polyadenylation signals are *double underlined*. The putative processing sites and the resulting B chain/C peptide/A chain are marked *(vertical lines)*

RNA blot hybridization

Total RNA was extracted from different human tissues (testis, brain, spleen, thyroid), size-fractionated by electrophoresis on 1% agarose gel containing formaldehyde, and transferred to nitrocellulose filters. Hybridization with the ^{32}P-labeled human cDNA was carried out overnight at 65° C, and filters were washed twice at 65° C to a final stringency of 0.2 × buffer A. Human α-actin cDNA was used for reprobing of the blots (Haunauer et al. 1983).

Results and discussion

Nucleotide sequence of the human Ley I-L

The human Ley I-L cDNA sequence of 743 bp consists of a 7-bp 5′-noncoding region, which is followed by a 393-bp open reading frame terminated by a TGA triplet, and a 343-bp untranslated 3′-end (Fig. 1). By means of primer extension analysis, the transcription start site was determined as being 14 bp upstream of the translation start site (data not shown). The first ATG codon of the open reading frame is within the consensus sequence ACCATGG, which was found to be optimal for the initiation of translation by eukaryotic ribosomes (Kozak 1989). The 3′-untranslated region of the cDNA includes two potential polyadenylation signals AATAAA, the last of which is located 11 bp upstream from the poly(A)tail (Fig. 1). Compared with a recently published nucleotide sequence of porcine Ley I-L cDNA, the nucleotide sequence of human Ley I-L cDNA shows a 73% similarity.

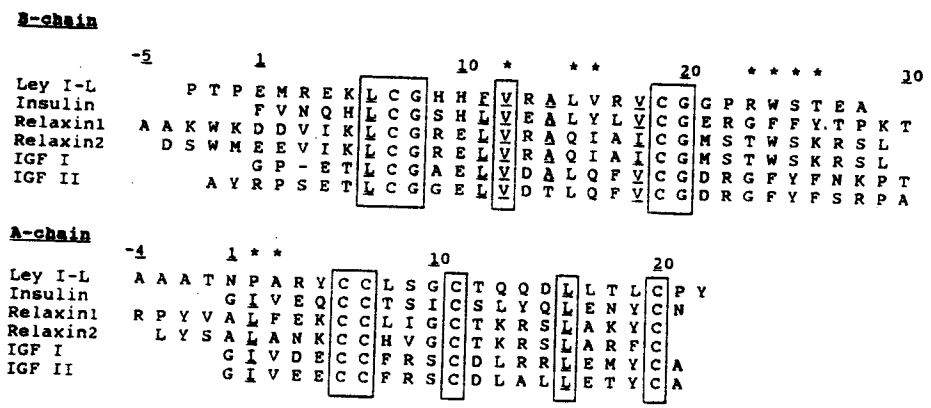

Fig. 2 Alignment of the amino acid sequences of human Leydig insulin-like peptide, insulin (Nicol and Smith 1960), relaxin (Hudson et al. 1984), insulin-like growth factor I (Jansen et al. 1983), and insulin-like growth factor II (Bell et al. 1984). Amino acids are *numbered* with respect to insulin. Identical residues are *boxed* and those that contribute to the hydrophobic core of insulin are *underlined*. Residues postulated to be involved in the binding of insulin to its receptor are marked with *stars*

Primary structure of human Ley I-L peptide

A 131-residue protein of 14.4 kDa is deducible from the open reading frame of the Ley I-L cDNA consensus sequence (Fig. 1). The amino acid sequence starts with a 24-residue signal peptide that comprises 16 hydrophobic amino acids and contains residues –12 (Leu) and –1 (Ala), which are conserved in all members of the insulin-related superfamily (Dull et al. 1984). The A and B chains containing the sequence features that define the insulin-related hormone superfamily (Blundell and Humbel 1980) have been identified in human Ley I-L by comparison with the corresponding sequence of human insulin (Nicol and Smith 1960).

Human pro Ley I-L contains the conserved amino acids of the B and A chains at the N- and C-terminal, respectively (Fig. 1). The two chains are separated by a long C peptide. The structure of pro Ley I-L shows greater similarity to that of proinsulin (Bell et al. 1979) and prorelaxin (Hudson et al. 1984) than to pro insulin-like growth factor I and II (Jansen et al. 1983; Bell et al. 1984). Pro insulin-like growth factors contain a small C peptide and two additional domains (D and E) located at the C-terminal. Because the pro Ley I-L is similar in structure to proinsulin and prorelaxin, it can be assumed that the mode of in vivo processing of the pro Ley I-L protein is analogous to the processing of proinsulin.

The proteolytic processing of the C peptide of proinsulin occurs at dibasic residues at the N- and C-terminal of the C peptide (Bell et al. 1979). Thus, cleavage at the B chain/C peptide is predicted to occur at the dibasic residue Arg-32/Arg-33. The cleavage at the C peptide/A chain junction of pro Ley I-L probably occurs after residue 81 (Arg) within a group of six dibasic residues (Fig. 1). If the predicted proteolytic sites of the human pro Ley I-L are correct, the active hormone will have a B chain of 31 residues and an A chain of 26 residues with a molecular weight of 6.25 kDa and an isoelectric point of 8.02, whereas the excised C peptide will have 50 residues.

A total of 19 and 17 amino acids in the predicted A and B chains of Ley I-L (Fig. 2) are identical to those in equivalent positions in human relaxin 1 (Hudson et al. 1984) and insulin (Nicol and Smith 1960) (35%), respectively. Although sequence similarity between human Ley I-L and human insulin is present in only 17 out of 49 aligned residues, the common conserved residues shown in Fig. 2 indicate that the three-dimensional conformation of human Ley I-L protein is similar to that of insulin. Ley I-L has retained not only the six cysteines but also the glycines at B8 and B20 (Fig. 2), and adopts unique torsion angles in the folded protein. In addition, the residues corresponding to those packed into the hydrophobic core of insulin (Blundell et al. 1972) are also hydrophobic in Ley I-L, with the exception of isoleucine at position A2 in insulin. Moreover, a histidine residue at B10 in insulin has been demonstrated as being essential for the binding of zinc in the hexamer structure (Bently et al. 1976), and is found at an equivalent position in Ley I-L (Fig. 2). A comparison between the complete amino acid sequence of human and porcine pro Ley I-L reveals a similarity of about 70%. When comparing only the sequences of the putative A and B chains, a similarity of about 92% and 80%, respectively, can be found. The sequence homology in the putative C peptide is only 59%.

Expression of the human Ley I-L gene

To analyze the expression of the human Ley I-L gene, blots prepared from the RNA of testis, brain, spleen, and thyroid gland were hybridized with the ^{32}P-labeled human Ley I-L1 cDNA fragment. A hybridization signal was only obtained with the RNA of testis. Rehybridization with α-actin revealed RNA integrity in all preparations. The mRNA for the human Ley I-L gene is synthesized as a molecule of approximately 0.9 kb (Fig. 3). The difference between the length of the cDNA (743 bp) and the mRNA is attributable to the poly (A) tail.

In boar, the Ley I-l gene is expressed exclusively in testicular Leydig cells; transcripts have been demonstrated in prenatal and postnatal testis (Adham et al. 1993). Because of the high structural similarity between

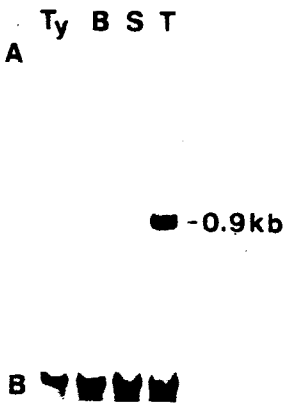

Fig. 3 A, B Analysis of Leydig insulin-like mRNA by RNA blot hybridization. **A** Total RNA (20 µg/lane) from human thyroid *(Ty)*, brain *(B)*, spleen *(S)*, and testis *(T)* was isolated, electrophoresed in 1% agarose/formaldehyde gel, and blotted onto nitrocellulose filter. The filter was hybridized with ^{32}P-labeled human Ley I-L cDNA. **B** The blot was subsequently washed and rehybridized with human α-actin cDNA

boar and human Ley I-L protein, the expression of the human Ley I-L gene is probably also confined to the Leydig cells, prenatally and postnatally. Leydig cells represent the endocrine tissue of the testis and provide hormones that play a role in spermatogenesis, and in the differentiation and maintenance of the male phenotype. The Ley I-L peptide could regulate the expression of genes that are involved in these developmental processes.

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