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MOLECULAR CLONING OF THE ACID-LABILE SUBUNIT OF THE RAT INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN COMPLEX

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SUMMARY : The insulin-like growth factors, IGF-I and IGF-II, circulate in both humans and rats as part of a 125-150 kDa complex comprising IGFs, the IGF binding protein IGFBP-3, and an acid-labile subunit. Clones encoding rat acid-labile subunit have been isolated from a rat liver cDNA library probed with a human acid-labile subunit cDNA. Two overlapping clones encode a leucine-rich protein of 576 amino acids preceded by a 27-residue signal sequence, with 78% homology to the human acid-labile subunit. Northern analysis of mRNA from adult rat brain, kidney, heart, lung, spleen, muscle and liver shows a major species of about 4.4 kb and minor bands of about 2 kb, 1.4 kb and 1 kb. The tissue distribution of this protein may therefore be wider than previously recognized.

It is well recognized that the insulin-like growth factor (IGFs) circulate in a growth hormone-dependent complex of 125-150 kDa [1-5]. In this complex, the acid-stable 45-kDa IGF-binding protein IGFBP-3, occupied by IGF-I or IGF-II, binds an 85-kDa glycoprotein, the acid-labile subunit (ALS) [6-7]. The existence of an acid-labile protein in the serum IGF complex was originally postulated by Furlanetto [6] and Hintz and Liu [8], and was confirmed after its purification from human serum in this laboratory [9]. It was found that ALS is not itself an IGF-binding protein, and has no effect on the binding of IGF-I or IGF-II to IGFBP-3 [9]. Since the 125-150 kDa complex is thought to cross the capillary barrier poorly, ALS may have an important role in regulating the access of circulating IGFs to the tissues [10].

Clones encoding the full amino acid sequence of human ALS have recently been isolated from human liver cDNA libraries [11]. A DNA construct encoding the full length of the protein, when tranfected into mammalian cells,

Abbreviations : IGF, insulin-like growth factor; IGFBP, IGF binding protein; ALS, acid-labile subunit.

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caused expression of a protein immunologically and functionally identical to serum ALS [11]. In the present study we report the isolation from a rat liver cDNA library of clones encoding the entire rat ALS sequence, and the detection of ALS mRNA in several rat tissues.

MATERIALS AND METHODS

Materials. Nucleic acid transfer membrane Hybond-N+, messenger affinity paper Hybond-mAP and Hyperfilm MP were obtained from Amersham Corp. (Bucks, UK). Taq DyeDeoxy Terminator Cycle sequencing kit was purchased from Applied Biosystems, Burwood, Victoria, Australia. RNA molecular weight markers were from Gibco BRL (Grand Island, NY). [^{32}P]dCTP was purchased from Dupont (Wilmington, DE). BamHI restriction endonuclease was from Boehringer Mannheim (Germany). All other chemicals were of analytical grade.

Tissue and RNA preparation. Adult Wistar-Furth rats, 8 weeks old, were euthanased under halothane anesthesia. Pools of brain, kidney, heart, lung, spleen, skeletal muscle and liver from 2 animals were rapidly frozen in liquid nitrogen and stored at -80°C. Total RNA was prepared by a single step acid guanidinium thiocyanate phenol-chloroform extraction protocol [12] and mRNA was selected by Hybond-mAP (messenger affinity paper) [13]. RNA was estimated by absorbance readings at 260 nm where 1 absorbance unit was assumed to be equal to 40 μg of RNA.

cDNA library screening. About 2,000,000 clones from a random primed rat liver cDNA library in the vector PUEX1 (donated by Dr. K. Stanley, Heart Research Institute, Camperdown, NSW, Australia) were screened with a ^{32}P-labeled human ALS probe [11]. Hybridization was at 65°C for 18 h in buffer containing 0.4 M sodium chloride, 0.05 M sodium phosphate, 5 mM EDTA, 0.5% SDS, 0.1% Ficoll, 0.1% polyvinylpyrolidone and 0.5 mg/ml sheared herring sperm DNA [14,15]. The filter was washed by incubating in 2 x SSC, 0.1% SDS at room temperature for 15 min, and then incubated in 0.2 x SSC, 0.1% SDS for 15 min at 60°C. The washed filters were exposed to Hyperfilm MP at -80°C overnight. Two positive clones were each rescreened to isolate the pure clones.

DNA sequencing. DNA sequences were determined using the fluorescent-labeled modified version of the dideoxy chain-termination method. Sequencing reactions were performed according to a cycle-sequencing protocol [16]; DNA (0.2 pmol), primer (3 pmol), dye terminators, nucleotides and Taq polymerase were reacted in a thermocycler for 15 cycles at 95°C for 30 sec, 60°C for 1 sec and 70°C for 1 min (followed by 15 cycles at 95°C for 15 sec and 70°C for 1 min). The reactions were pelleted with ethanol and electrophoresed on an 370A DNA sequencer (Applied Biosystems).

Northern blotting analyses. Six micrograms of mRNA were electrophoresed on a 2.2 M formaldehyde agarose gel and then transferred onto Hybond N+ nylon membrane filters. Hybridization was performed with a [^{32}P]dCTP random labeled probe that encodes the amino-terminal 518 amino acids of rat ALS, in a solution of 20% formamide at 42°C for 18 h [14]. The filters were washed with vigorous agitation in 1 x SSC, 0.1% SDS for 15 min at 60°C and set up for autoradiography using Hyperfilm MP for 72 h at -80°C. Size estimation of the hybridizing band was obtained from the relative migration rates of a commercially available RNA ladder.

RESULTS AND DISCUSSION

Clones encoding rat ALS were isolated by screening a rat liver cDNA library with a human ALS probe. Three positive clones out of two million independent clones were detected in two screenings. Fig. 1 shows a schematic representation of two of these clones. One clone (Rals.1) was found to encode the carboxy-terminal 545 amino acids, followed by a noncoding DNA sequence of approximately 1.7 kb. The other clone (Rals.2) encodes the amino-terminal 518 amino acids of rat ALS preceded by an noncoding sequence of approximately 380 bases. The full cDNA and amino acid sequence of rat ALS, derived from clones Rals.1 and Rals.2, are shown in Fig. 2.

By analogy with the human ALS sequence [11], the methionine codon at nucleotides #1-3 of the cDNA sequence shown in Fig. 2 is likely to be the initiation site of translation. As previously discussed [11], the sequence surrounding this ATG (ACAATGG) in the rat sequence is in good agreement with the consensus sequence (A[G]XXATGG) for the start of translation in eukaryotes proposed by Kozak [17]. Furthermore, another ATG, which is found in the human cDNA sequence 7 bases before the putative initiation codon, is not present in the rat sequence. Thus, the cDNA encoding rat ALS predicts a mature protein of 576 amino acids (64.1 kDa) preceded by a 27-residue hydrophobic peptide.

Comparison of the mature rat and human ALS amino acid sequences reveals 125 amino acid substitutions and two deletions (both towards the carboxy-terminus) in the rat sequence, i.e. the human and rat sequences are 78% homologous. Like the human sequence, some 80% of rat ALS is composed of 19-20 leucine-rich repeats of 24 amino acids, beginning at about residue 40 (Fig. 3). As in the human protein, these repeats have a concensus sequence which is also found in several other leucine-rich glycoproteins known to participate in protein-protein or protein-membrane interactions [18].

Northern blot hybridization shows that a major species of about 4.4 Kb and minor bands of about 2.0 Kb, 1.4 Kb and 1.0 Kb are found in adult rat brain, kidney, heart, lung, spleen, muscle and liver (Fig. 4). While human liver ALS mRNA species of similar sizes have been described, the relative intensities of

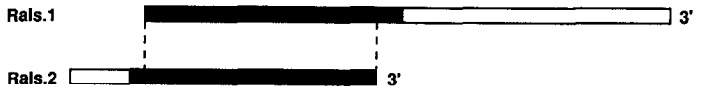

Fig. 1. Schematic representation of the two rat ALS cDNA clones (Rals.1 and Rals.2) which together cover the full (1.81 kb) coding sequence. The coding regions are represented by filled boxes; the 5' and 3' noncoding regions are represented by open boxes. Dashed lined indicate the 1.46 kb of overlapping nucleotides.

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-100 GCTGCCAGCTACAGGCAGTGGGGAAATCCACAGGGCAGCAGCTGTATTGTAGACGGCCCTTGCTCACTGCCTGCCTGCAGCCAGCTCTGTACAAGGAACA  -1
   1 ATGGCCCTGAGGACAGGAGGCCCAGCCCTGGTGGTGCTTCTGGCTTTCTGGGTGGCACTGGGCCCCTGTCACCTGCAGGGGACAGATCCCGGAGCGTCGG  100
     M  A  L  R  T  G  G  P  A  L  V  V  L  L  A  F  W  V  A  L  G  P  C  H  L  Q  G  T  D  P  G  A  S
                                                                                          1
 101 CAGATGCCGAGGGCCCCCAGTGCCCCGTCGCCTGTACCTGCAGCCATGATGACTACACAGATGAGCTCAGCGTCTTTTGCAGTTCAAAGAACCTCACACA  200
     A  D  A  E  G  P  Q  C  P  V  A  C  T  C  S  H  D  D  Y  T  D  E  L  S  V  F  C  S  S  R  N  L  T  H
 201 TCTGCCTGATGACATCCCAGTCAGCACCAGAGCCCTGTGGCTTGATGGCAACAACCTCTCTTCTATCCCCTCAGCGGCCTTCCAGAACCTGTCCAGCCTG  300
       L  P  D  D  I  P  V  S  T  R  A  L  W  L  D  G  N  N  L  S  S  I  P  S  A  A  F  Q  N  L  S  S  L
 301 GACTTTCTCAACCTGCAGGGCAGCTGGCTGAGGAGCCTGGAGCCACAGGCACTGCTGGGGCTGCAGAACCTCTACTATCTGCACCTGGAACGGAACCGGC  400
     D  F  L  N  L  Q  G  S  W  L  R  S  L  E  P  Q  A  L  L  G  L  Q  N  L  Y  Y  L  H  L  E  R  N  R
                                                                                       100
 401 TCCGGAACCTCGCCGTGGGCTTGTTCACACACACACCGAGTCTGGCTTCACTCAGCCTGAGCAGCAACCTCTTGGGCCGGCTGGAGGAAGGGCTGTTCCA  500
     L  R  N  L  A  V  G  L  F  T  H  T  P  S  L  A  S  L  S  L  S  S  N  L  L  G  R  L  E  E  G  L  F  Q
 501 GGGCCTCAGTCACCTTTGGGACCTCAACCTGGGTTGGAACAGTCTAGTGGTCCTGCCTGACACAGTGTTCCAGGGACTGGGCAACCTCCACGAGCTGGTG  600
       G  L  S  H  L  W  D  L  N  L  G  W  N  S  L  V  V  L  P  D  T  V  F  Q  G  L  G  N  L  H  E  L  V
 601 CTGGCTGGCAACAAACTGACTTACCTGCAGCCTGCGCTCTTCTGTGGCTTGGGCGAGCTGCGGGAGCTGGATCTGAGCAGGAACGCACTCCGAAGCGTCA  700
       L  A  G  N  P  L  T  Y  L  Q  P  A  L  F  C  G  L  G  E  L  R  E  L  D  L  S  R  N  A  L  R  S  V
                                                                                       200
 701 AAGCTAACGTCTTTGTACATTTGCCCAGGCTGCAGAAGCTGTACCTGGACCGGAACCTCATTACAGCCGTGGCCCCTGGTGCCTTTCTGGGCATGAAGGC  800
     K  A  N  V  F  V  H  L  P  R  L  Q  K  L  Y  L  D  R  N  L  I  T  A  V  A  P  R  A  F  L  G  M  K  A
 801 CCTGCGTTGGCTGGACCTGTCGCACAACCGCGTGGCTGGCCTCATGGAGGACACCTTCCCAGGCCTGCTGGGCCTGCACGTCCTGCGCCTGGCACACAAT  900
       L  R  W  L  D  L  S  H  N  R  V  A  G  L  M  E  D  T  F  P  G  L  L  G  L  H  V  L  R  L  A  H  N
 901 GCGATCGCTAGCTTGCGGCCGCGCACTTTCAAAGACCTGCACTTCCTGGAGGAACTGCAGCTGGGCCACAATCGAATCAGGCAGCTCGGGGAGAGGACAT 1000
       A  I  A  S  L  R  P  R  T  F  K  D  L  H  F  L  E  E  L  Q  L  G  H  N  R  I  R  Q  L  G  E  R  T
                                                                                       300
1001 TCGAGGGCCTGGGGCAGCTGGAGGTGCTGACGCTCAATGACAACCAGATCACTGAGGTCAGGGTGGGCGCCTTCTCTGGCCTTTTCAATGTGGCGGTTAT 1100
     F  E  G  L  G  Q  L  E  V  L  T  L  N  D  N  Q  I  T  E  V  R  V  G  A  F  S  G  L  F  N  V  A  V  M
1101 GAATCTCTCCGGCAACTGTCTGAGGAGCCTCCCGGAGCGGGTGTTTCAGGGTCTGGACAAACTGCACAGCCTGCACCTAGAGCACAGCTGCCTGGGTCAC 1200
       N  L  S  G  N  C  L  R  S  L  P  E  R  V  F  Q  G  L  D  K  L  H  S  L  H  L  E  H  S  C  L  G  H
1201 GTCCGCCTGCACACTTTTGCTGGCCTCTCAGGGCTGCGCAGGCTCTTCCTCAGGGACAACAGCATCTCCAGCATCGAAGAACAGAGCCTGGCAGGGCTTT 1300
       V  R  L  H  T  F  A  G  L  S  G  L  R  R  L  F  L  R  D  N  S  I  S  S  I  E  E  Q  S  L  A  G  L
                                                                                       400
1301 CGGAGCTCCTGGAACTGGATCTTACTACCAACCGCCTCACACATCTGCCCCGCCAGCTCTTCCAGGGCCTCGGCCACCTGGAGTACCTGCTTCTCTCCTA 1400
     S  E  L  L  E  L  D  L  T  T  N  R  L  T  H  L  P  R  Q  L  F  Q  G  L  G  H  L  E  Y  L  L  L  S  Y
1401 CAACCAACTGACGACTTTATCCGCGGAGGTCCTGGGCCCTCTGCAGCGGGCCTTCTGGTTGGATATCTCACACAACCACCTGGAGACGCTGGCCGAAGGC 1500
       N  Q  L  T  T  L  S  A  E  V  L  G  P  L  Q  R  A  F  W  L  D  I  S  H  N  H  L  E  T  L  A  E  G
1501 CTTTTCTCATCTCTGGGGCGCGTTCGCTACCTCAGCCTCAGGAATAACTCCTTGCAGACCTTTTCACCACAGCCCGGCCTGGAGCGCCTGTGGCTTGATG 1600
       L  F  S  S  L  G  R  V  R  Y  L  S  L  R  N  N  S  L  Q  T  F  S  P  Q  P  G  L  E  R  L  W  L  D
                                                                                       500
1601 CCAACCCCTGGGACTGCAGCTGTCCCCTCAAGGCGCTTCGAGACTTTGCCCTGCAGAACCCTGGTGTTGTCCCCCGCTTTGTTCAGACTGTCTGTGAGGG 1700
     A  N  P  W  D  C  S  C  P  L  K  A  L  R  D  F  A  L  Q  N  P  G  V  V  P  R  F  V  Q  T  V  C  E  G
1701 GGACGACTGCCAGCCGGTGTACACCTACAACAATATCACTTGCGCTGGCCCCGCCAACGTCTCGGGCCTCGACCTAAGAGACGTTAGTGAAACACATTTT 1800
       D  D  C  Q  P  V  Y  T  Y  N  N  I  T  C  A  G  P  A  N  V  S  G  L  D  L  R  D  V  S  E  T  H  F
1801 GTGCACTGCTGACACTGGCTACTTACTGGCCCGGTCTGGCCGAACACTGTCTCATGGCCAGGACGGTGTCTCATTGTTAACAGAATAAGCTGGCTCTCAA 1900
       V  H  C  O
             576
1901 ATTCCTACCCATCTCTAGGGGACAGGTCCTGGCTGCTCACTTCCTGGAAGCAGGCTGTACTGGAAGCTATGTGGCCTAGAAAGGGTGGGCTCAGGCCAAG 2000
2001 TGTCCAAGGGCCCAAAGGAGGGAGGTGCTCGCTGAATTTAAGCATATTAGTCAGCGGAGGAAAAGAAACTAACCAGGATTCCCTCAGTAA
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Fig. 2. Nucleotide and deduced protein sequence of rat ALS. Nucleotides are numbered in the margins; nucleotide #1 begins the translated sequence. Amino acids are numbered in bold, beneath the one-letter amino acid sequence; residue #1 begins the sequence of the mature protein.

these bands in the rat tissues examined is quite different, with the 4.4 kb band the most intense, rather than the 2.0 kb band as seen in human liver [11]. At least two separate tissue pools for each rat tissue were extracted and analyzed, with similar results. To date, no studies of ALS synthesis by human cells have been reported. In the rat, hepatocyte cultures have been shown to synthesize ALS under growth hormone control [19], but other sites of synthesis are unknown. The demonstration of ALS mRNA in several rat tissues raises the possibility of ALS synthesis in a variety of different sites.

The origin of the multiple mRNA species is unknown. Possibly they may result from mRNA degradation, as the destabilizing sequences ATTTA [20] is found at nucleotides #2036-2040 in the 3'-untranslated region of rat ALS cDNA as shown in Fig. 2. This sequence may be recognized by an RNA-binding

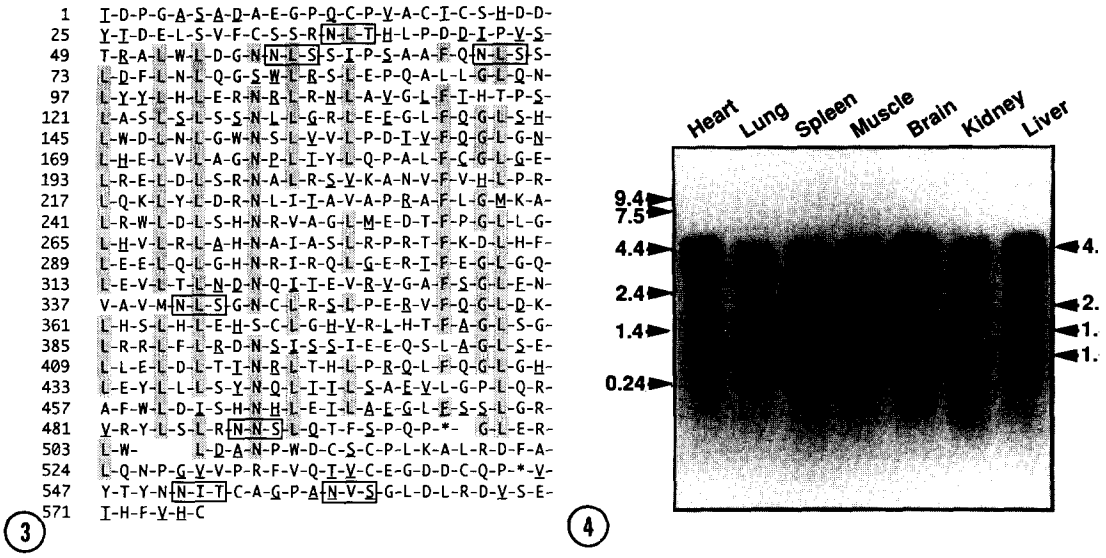

Fig. 3. Amino acid sequence of rat ALS, aligned to indicate the leucine-rich repeats. Highly-repeated residues are shaded; potential N-glycosylation sites are boxed. Differences from the human ALS sequence are underlined; deletions from the human sequence are indicated by asterisks; and spaces, where present, are added to improve alignment.

Fig. 4. Northern blot analysis of ALS mRNA in rat tissues. Six micrograms of mRNA was prepared from adult rat heart, lung, spleen, skeletal muscle, brain, kidney, and liver, and analyzed by Northen blotting with a rat ALS cDNA as described in the text. The numbers on the left indicate the size in kb of RNA standards, and on the right, the estimated size of the detected bands.

protein involved in the specific degradation of transiently expressed mRNAs, as the 3'-untranslated regions of mRNAs are particularly accessible to nucleases [21]. Future studies will address the differential tissue-specific or hormonal regulation of these mRNAs.

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