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Ulip6, a novel unc-33 and dihydropyrimidinase related protein highly expressed in developing rat brain

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Abstract **Here, we report the identification of Ulip6, a novel unc-33 and dihydropyrimidinase related protein that belongs to the Ulip/CRMP protein family. Ulip6 was found in a yeast two-hybrid screen using the neuronal glycine transporter GlyT2 as bait. The rat and human Ulip6 sequences are highly homologous and most closely related to the liver enzyme dihydropyrimidinase (Ulip5). Northern and Western analysis of rat tissues revealed that the distribution of the Ulip6 mRNA and protein resembles those of brain-type Ulip proteins. Like Ulip1–4, Ulip6 is highly expressed in embryonic and early postnatal brain and spinal cord. These findings are consistent with Ulip6 having a function in neuronal differentiation and/or axon growth.** **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Glycine transporter 2;
Unc-33 like phosphoprotein (Ulip);
Collapsin response mediator protein (CRMP);
Unc-33; Dihydropyrimidinase;
Neuronal differentiation

1. Introduction

The development of neuronal networks requires the outgrowth of axons from postmitotic neurons towards their target cells to allow the establishment of functional synaptic connections. Axonal growth depends on a specialized subcellular structure at the tip of the extending neurite, the neuronal growth cone. Besides a highly specialized cytoskeleton, the growth cone contains a number of plasma membrane proteins and receptors that are involved in neurotransmitter metabolism and recognition of extracellular guidance cues during the process of axonal pathfinding. Among these are receptors for attracting and repelling signaling molecules, such as the netrins, collapsins/semaphorins and ephrins [1], as well as transporters for neurotransmitters, like serotonin [2] and excitatory and inhibitory amino acids [3,4]. All these membrane proteins have been reported to be enriched in the growth cone and may also be found in mature presynaptic terminals.

In an attempt to identify the intracellular machinery that targets these membrane proteins to the axonal compartment, we have employed a yeast two-hybrid screen with a transporter for the inhibitory amino acid glycine, the Na^+/Cl^- dependent glycine transporter GlyT2. This transporter has been demonstrated to be highly concentrated in the growth cone of developing spinal neurons [3] and is characterized by a unique N-terminal cytoplasmic domain of 201 amino acids [5]. Unexpectedly, our screen with this domain identified a novel gene product that belongs to a family of proteins which have been implicated in the signaling cascades mediating axonal guidance. In this report, we present the sequences of this protein, termed Ulip6, from rat and human and show that it is highly expressed in the embryonic and early postnatal rat nervous system.

2. Materials and methods

2.1. Identification of rat and human Ulip6 cDNAs

Yeast two-hybrid screening was performed with the DupLEX-A™ yeast two-hybrid system on a cDNA library constructed from adult rat brain in pJG4-5 (OriGene). As a bait, the N-terminal region of rat GlyT2 (residues 1–201) [5] inserted into pGilda (OriGene) was used. Screening of 2×10^7 clones resulted in the isolation of 48 blue colonies. Out of these, seven clones harboring the same Ulip6 cDNA were isolated. To identify the missing 5′ region, we performed PCR on an adult rat brain cDNA library (Clontech) cloned in the pGAD10 vector using the sequence 5′-CTTGGTCCCGTGGTAGAAGTCG-3′ corresponding to nucleotides 363–384 of the Ulip6 cDNA and the adapter primer 5′-CTATTCGATGATGAAGATACCCCACCAA-ACCC-3′ complementary to the vector sequence. The resulting product was re-amplified using a nested primer 5′-CAGTCATCGTTCAC-CACCTTGCC-3′ deduced from the Ulip6 cDNA (nucleotides 166–188) and the adapter primer specified above. The final product was cloned into the pCR 2.1-TOPO vector for sequencing (Invitrogen). All DNA sequences were verified by dideoxy-sequencing of both strands.

To isolate the human Ulip6 cDNA, cDNA was synthesized from a human spinal cord poly(A)$^+$RNA (Clontech) using a first-strand cDNA synthesis kit (Pharmacia Biotech). This cDNA served then as template for PCR amplification with the primers 5′-CGCAG-CATCCTGGAGGAGAA-3′ and 5′-GCAGCTTCGGCTGGAGAG-TT-3′ deduced from the human ESTs AI571188 and AI369696. These ESTs were identified by BLAST search in the DDBJ web site and correspond to the putative 5′ and 3′ untranslated regions of the human Ulip6 sequence.

The rat and human Ulip6 nucleotide sequences are deposited in the EMBL/GenBank database under accession numbers AJ131436 and AJ251275.

2.2. Northern analysis

RNA isolation from various rat tissues was performed using the RNeasy kit (Qiagen). Total RNA (11 μg per lane) was analyzed by Northern blotting as described previously [6]. As hybridization probes, both a 1.7 kb fragment amplified by PCR from the coding sequence (nucleotides 127–1833) and a 3.0 kb *Pst*I/*Xho*I fragment corresponding to the 3′ untranslated region (nucleotides 1895–4845) of the rat Ulip6 cDNA were labelled with a randomly primed DNA labelling kit (Boehringer Mannheim). A 1.1 kb fragment of the mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (Clontech) was used as internal control. After hybridization, membranes were washed twice with 0.1×SSC and 0.1% (w/v) SDS at 60°C for 20 min, and autoradiography was performed with Biomax™ MR X-ray film (Kodak) at −80°C for 7 days.

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2.3. Antibodies

Two peptides (peptide 1: KLVQREKTLKVRGVDRTPC; peptide 2: DTPTRPVTRHGGMRDLC) corresponding to positions 474–491 and 513–528, respectively, of rat and human Ulip6 were synthesized (Fig. 1A) and conjugated to keyhole limpet hemocyanin to allow immunization of rabbits (Biotrend). Polyclonal anti-Ulip6 sera were collected over 2 months after several booster injections with both antigens. A mouse monoclonal antibody (12CA5) against hemagglutinin (HA) was purchased from Boehringer Mannheim.

2.4. Expression constructs

An HA epitope was inserted into the Ulip1–6 sequences after the initiator methionine by using PCR with the following cDNAs as templates: mouse Ulip1–4 cDNAs, kindly provided by Dr. A. Sobel, Paris, France; rat Ulip5 (dihydropyrimidinase), donated by Drs. K. Matsuda and N. Tamaki, Kobe, Japan; and rat Ulip6 isolated here. The products were subcloned into the eukaryotic expression vector pBK-CMVΔlac (Stratagene) [7] for use in transfection experiments.

2.5. Recombinant expression of Ulip proteins in HEK 293 cells and Western blotting

The culture conditions for human embryonic kidney 293 (HEK 293) cells (ATCC#CRL-1573) and the protocols used for transfections have been described in detail [8]. For the detection of the different Ulip proteins in transfected cells, and of Ulip6 in various rat tissues, homogenates were prepared according to standard procedures and separated by 10% SDS–PAGE followed by Western blotting [9]. Antigen/antibody complexes were visualized using enhanced chemiluminescence (Pierce) and Kodak Biomax film. Protein concentration was measured by the method of Bradford [10] using bovine serum albumin as a standard.

3. Results and discussion

3.1. Isolation of rat and human Ulip6 cDNAs and structure of the deduced proteins

In an attempt to isolate proteins that interact with the neuronal glycine transporter GlyT2 [5], we used the yeast two-hybrid system to screen a rat brain cDNA library using the large cytoplasmic N-terminal region of GlyT2 as a bait. This identified a cDNA clone which contained an insert of 4821 bp. The missing 5′ region was completed by PCR (see Section 2); this led to a 24 nucleotide extension of the original clone. The protein sequence deduced from the complete cDNA of 4845 bp is shown in Fig. 1. The encoded protein displays highly significant homologies to all known members of the unc-33 [11] like phosphoprotein (Ulip)/collapsin response mediator protein (CRMP) family [12–19], and hence was termed Ulip6.

A BLAST search with the rat Ulip6 sequence identified several homologous human ESTs (AI571188, N51749, AA350414, AI498064, AI369696). Using primers corresponding to the predicted non-coding regions of these human ESTs, we isolated a cDNA of 1745 bp encoding the human Ulip6 protein. The deduced sequences of the human and rat proteins show 97.7% identity and are composed of 564 amino acids (calculated molecular weights in rat, 61.5 kDa; human, 61.4 kDa). Both proteins share homology values of 37% with the *Caenorhabditis elegans* unc-33 protein. However, the Ulip6

A

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Rat     1  MLANSASVRILIKGGKVVNDDCTHEADVYIENGIIQQVGRELMIPGGAKVIDATGKLVIPGGIDTSTHFHQTFMNATCVDDFYHGTKAALVGGTTMIIGH
           ****************************************************************************************************
Human   1  MLANSASVRILIKGGKVVNDDCTHEADVYIENGIIQQVGRELMIPGGAKVIDATGKLVIPGGIDTSTHFHQTFMNATCVDDFYHGTKAALVGGTTMIIGH

      101  VLPDKETSLVEAYEKCRALADPKVCCDYALHVGITWWAPKVKAEMETLVREKGVNSFQMFMTYKDLYMLRDSELYQVFHACRDFGAIPRVHAENGELVAE
           **********.******.***********************************************************.***.*.***.************
      101  VLPDKETSLVDAYEKCRGLADPKVCCDYALHVGITWWAPKVKAEMETLVREKGVNSFQMFMTYKDLYMLRDSELYQVLHACKDIGAIARVHAENGELVAE

      201  GAKEALDLGITGPEGIEISHPEELEAEATHRVITIANRTHCPIYLVNVSSISAGDVIAAAKMQGKVVLAETTNAHATLTGLHYYHQDWSHAAAYVTVPPL
           *******************.****************************************************.***************************
      201  GAKEALDLGITGPEGIEISRPEELEAEATHRVITIANRTHCPIYLVNVSSISAGDVIAAAKMQGKVVLAETTTAHATLTGLHYYHQDWSHAAAYVTVPPL

      301  RLDTNTSTYLMSLLANDTLNIVASDHRPFTTKQKAMGKEDFTKIPHGVSGVQDRMSVVWERGVVGGKMDENRFVAVTSSNAAKILNLYPRKGRIIPGADA
           *********************************************************.*********************.***.****************
      301  RLDTNTSTYLMSLLANDTLNIVASDHRPFTTKQKAMGKEDFTKIPHGVSGVQDRMSVIWERGVVGGKMDENRFVAVTSSSAAKLLNLYPRKGRIIPGADA

      401  DVVVWDPEATKTISASTQVQGGDFNLYENMRCHGVPLVTISRGRVVYENGVFMCAEGTGKFCPLRSFPDIVYKKLVQREKTLKVRGVDRTPYLGDVAVVV
           *********************************************************************.******************************
      401  DVVVWDPEATKTISASTQVQGGDFNLYENMRCHGVPLVTISRGRVVYENGVFMCAEGTGKFCPLRSFPDTVYKKLVQREKTLKVRGVDRTPYLGDVAVVV

      501  NPGKKEMGTPLADTPTRPVTRHGGMRDLHESSFSLSGSQIDDHVPKRASARILAPPGGRSSGIW
           .***************************************************************
      501  HPGKKEMGTPLADTPTRPVTRHGGMRDLHESSFSLSGSQIDDHVPKRASARILAPPGGRSSGIW
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B

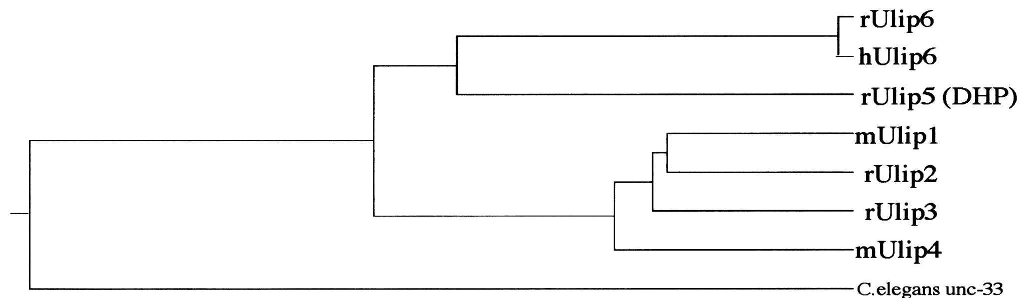

Fig. 1. A: Primary structures of rat and human Ulip6. Asterisks and dots denote identical and homologous amino acid residues. The thin and thick lines indicate peptide sequences used for immunization (anti-pep-1 and anti-pep-2, respectively). B: Phylogenetic tree of the unc-33 protein family. The relationship between mammalian Ulip (m, mouse; r, rat; h, human) and *C. elegans* unc-33 protein sequences was analyzed by the unweighted pair-group method-assisted (UPGMA) procedure [24] using GENETYX software.

polypeptides are more homologous to the mammalian liver enzyme dihydropyrimidinase (DHP or Ulip5) than to the neuronal Ulip proteins 1–4 (Fig. 1B). An overall sequence identity of 57% is found with Ulip5 (DHP) [17,18], whereas identities of only 50–51% are seen with Ulip1–4 [12–16,19]. Homology values differ significantly within the Ulip domains I–V postulated by Byk et al. [19]. The most highly conserved region (90–100% identity with the mammalian Ulip1–5) corresponds to subdomain III d′, a 32 amino acid motif (residues 374–405 in Ulip6) that is shared by two recently identified Ulip proteins from *C. elegans*, the *Pseudomonas putida* bacterial hydantoinase, Ulip5 (DHP) and the mammalian Ulip1–4 polypeptides [19]. These latter proteins have been proposed to be part of the signaling cascade that directs axon growth through guidance molecules of the collapsin/semaphorin family [11,12].

3.2. *Ulip6 mRNA and protein are highly expressed in the embryonic and early postnatal nervous system*

To unravel the site of expression of Ulip6, we examined the distribution of the Ulip6 mRNA and protein in neuronal and non-neuronal tissues. Of the latter, liver and kidney are known to be primary sites of Ulip5 (DHP) expression. Fig. 2 shows a Northern blot analysis of Ulip6 transcript distribution. The blot probed with the coding region of the rat Ulip6 cDNA revealed a broad hybridizing band of 4.5–5.2 kb in neuronal (brain cortex, cerebellum, spinal cord) but not non-neuronal (heart, lung, liver, kidney) tissues. Notably, a strong signal was only seen at embryonic day 18 (E18) and the first day postnatally (P1), but not in adult nervous tissue. The same mRNA was also detected when using a probe corresponding to the 3′ non-coding region of the cDNA (data not shown); its size is in excellent agreement with the size of the full-length cDNA clone (4845 bp) isolated from rat. A possible explanation for the diffuse nature of the Ulip6 mRNA may be the existence of multiple poly(A)$^+$ addition signals. An ATTAAA sequence, positioned at nucleotides 4825–4830, may be used in addition to an AATAAA tract found at nucleotides 4412–4417 (Fig. 2B). Notably, a poly-(GT) sequence is found about 60 bp 3′ of the potential upstream polyadenylation site. Such GT-rich sequences have been proposed to bind components of the transcriptional machinery, such as cleavage stimulation factors [20]. In conclusion, the Ulip6 mRNA is predominantly expressed in embryonic and neonatal neuronal tissue, and its level is down-regulated during postnatal development. Similar expression patterns have also been described for the other neuronal Ulip proteins, Ulip1–4 [13–16,19].

To further corroborate the selective expression of Ulip6 in the developing rat brain, we raised specific antibodies against peptides deduced from amino acids 474–491 and 513–528 of the rat and human proteins. Fig. 3A shows that the resulting antibodies detected specifically Ulip6, but not mouse Ulip1–4, upon heterologous expression of the HA-tagged polypeptides in HEK 293 cells. Thus, our antisera were highly selective for Ulip6 (the lack of mouse Ulip1–4 immunoreactivities cannot be attributed to the species difference, since Ulip sequences are >99% conserved between rat and mouse). Using these antibodies, we again detected Ulip6 only in neuronal tissues including cortex, cerebellum and spinal cord. Also, protein levels were high at E18 and P1, but dropped to very low levels in the adult nervous system, thus corroborating our data on mRNA distribution shown in Fig. 2A.

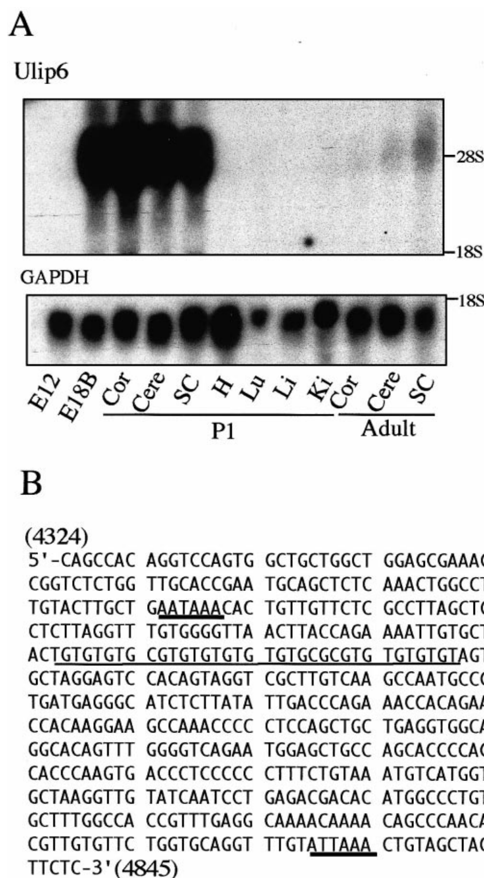

Fig. 2. A:Distribution of Ulip6 transcripts in various rat tissues. Northern blotting was performed as described in Section 2. After hybridization to the Ulip6 probe, rehybridization disclosed the GAPDH mRNA as a loading control. Abbreviations are: E12, whole embryo day 12; E18B, embryonic brain day 18; Cor, brain cortex; Cere, cerebellum; SC, spinal cord; H, heart; Lu, lung; Li, liver; Ki, kidney; P1, 1 day after birth. B: Partial 3′ untranslated sequence of the rat Ulip6 cDNA. The thick lines indicate the two poly(A)$^+$ addition signals. A unique GT-rich region found about 60 bp after the first polyadenylation site is also underlined.

3.3. *Conclusion*

In this study, we describe a novel unc-33 related protein from rat and human, named Ulip6. Its primary sequence is most similar to Ulip5 (DHP) found in liver and kidney, but its expression pattern characterized by high mRNA and protein levels in differentiating neuronal tissue resembles that of the neuronal Ulip1–4. This suggests that Ulip6 may play an important role in axon growth and neuronal differentiation, as proposed for the Ulip1–4 proteins [12,19,21]. It should, however, be noted that the structural similarity between Ulip6 and DHP (Ulip5) may be indicative of related functions. In other words, Ulip6 might display DHP activity although no such activity has been detected in brain homogenates using a standard DHP assay [22]. Also, Ulip5 (DHP) is known to be a homo-oligomeric trimer in its native conformation [23], whereas other Ulip proteins such as Ulip2 and Ulip3 have been reported to form hetero-oligomers. Future studies should show whether Ulip6 may hetero-oligomerize with one of the Ulip1–4 proteins, and whether it indeed serves in intracellular signalling processes that control axon growth and neuronal differentiation.

During submission of this manuscript, the rat Ulip6 se-

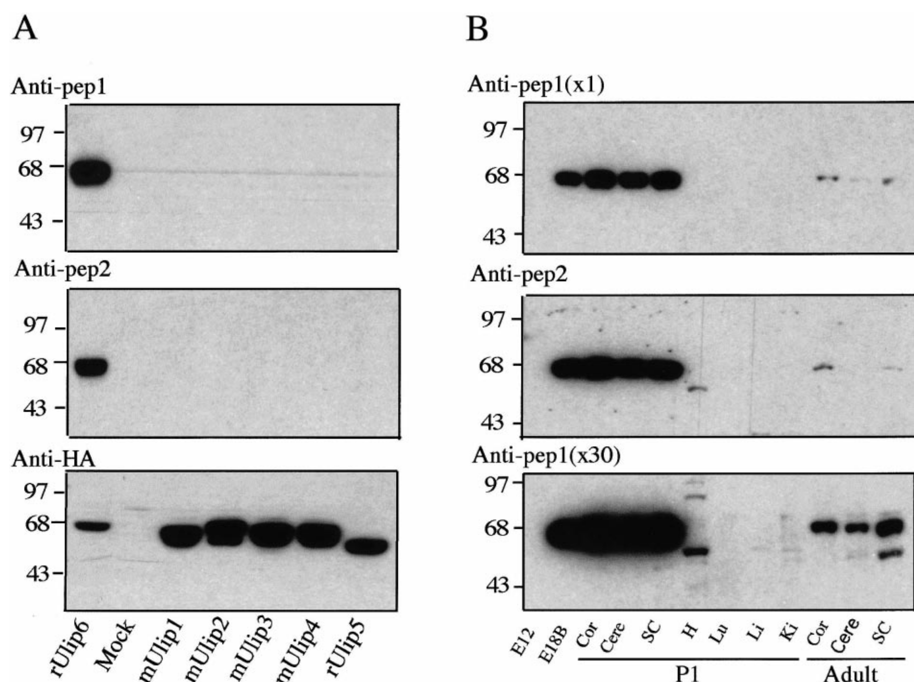

Fig. 3. Western blot analysis. A: Specificity of anti-peptide antibodies against Ulip6. Lysates of HEK 293 cell transfected with the indicated constructs were used for Western blotting with the respective antibodies. Expression levels of each construct were monitored by reprobing the membrane with an anti-HA antibody (lower left panel). B: Distribution of Ulip6 protein in various rat tissues. The labelling of lanes is as in Fig. 2A; 5 μg of protein was applied per lane. In case of anti-pep-1, the X-ray film shown in the lower right panel was exposed 30 times longer than that in the upper right panel. The positions of molecular weight markers (in kDa) are indicated.

quence has also been reported in electronic form as 'CRMP3-associated molecule' (CRAM) by Inatome et al. (J. Biol. Chem., papers in press, 10.1074/jbc.M910126199). Notably, the deduced CRAM protein sequence differs from rat Ulip6 by lacking one of the two cysteine residues at positions 125/126 that are highly conserved throughout the Ulip family.

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