LOCALIZATION AND DEVELOPMENTAL CHANGES
OF mRNA FOR CDP-DIACYLGLYCEROL SYNTHASE
IN RAT CENTRAL NERVOUS SYSTEM

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Abstract : CDP-diacylglycerol synthase (CDS) catalyzes the reaction of CTP and phosphatidic acid to
form CDP-diacylglycerol and inorganic pyrophosphate. In this study, we isolated and sequenced a rat
CDS clone that encodes an amino acid sequence highly homologous to the human CDS sequence. With
the use of the cloned cDNA, we investigated the distribution of the mRNA in the adult rat central
nervous system by in situ hybridization. The CDS transcript was widely distributed throughout the brain,
although signal intensities were somewhat variable. The mRNA was moderately expressed in the mitral
cell layer of the olfactory bulb, piriform cortex, neocortex layer V/VI, dentate gyrus, CA1-4 regions of
hippocampus, Purkinje cell layer of the cerebellum, and dorsal root ganglion. In neocortex layers II/III
and IV, amygdala, hypothalamus, and granule cells of the cerebellum were relatively few, but significant
levels of the CDS transcript were observed. We also studied the developmental changes in the expression
of the CDS mRNA in the olfactory bulb, hippocampus, cerebellum and spinal cord by the method of
reverse transcription-polymerase chain reaction. Dramatic increases in the expression of the CDS mRNA
were seen during the early stage of postnatal growth.

Key words : CDP-diacylglycerol synthase, developmental change, mRNA localization, mRNA
synthesis, rat brain.

INTRODUCTION

CDP-diacylglycerol (CDP-DAG), which is considered as an activated form of phosphatidic acid (PA),
plays a central role in the biosynthesis of glycerophospholipids in both prokaryotic and eucaryotic organ-
isms1&4). It is a required branch point intermediate in the synthesis of phosphatidylycerine, phosphatidylinositol (PI), phosphatidylglycerol, and cardioliipin. The very low tissue levels of CDP-DAG5) suggest that the reaction catalyzed by its biosynthetic enzyme, CDP-DAG synthase (CDS)[EC 2.7.7.41],
may be rate-limiting for some downstream lipid synthesis and thus may be a potential site of regulation.

The phosphoinositide cycle produces intracellular second messengers that mediate various cellular responses to stimuli such as growth factors and neurotransmitters6&7). After phosphatidylinositol 4,5-bis-
phosphate (PIP2) is hydrolyzed by phospholipase C (PLC) into the two second messengers inositol 1, 4,
5-triphosphate and diacylglycerol (DAG), it must be resynthesized. DAG is phosphorylated to produce PA by DAG kinase. PA is converted to CDP-DAG by CDS. CDP-DAG is then converted to PI by PI
synthase. Finally, PI is sequentially phosphorylated by PI 4-kinase and PI 4-phosphate 5-kinase to form PIP2. Thus CDS functions not only in the de novo synthesis, but also in the resynthesis of phos-
phoinositides. The importance of CDS in the signal transduction has been strengthened in a recent study by Wu et al., showing that overexpression of a photoreceptor cell-specific isoform of CDS in Drosophila increases the amplitude of the light response.

Gene cloning is an essential prerequisite for many regulatory and structural studies. The cloning of a gene encoding CDS was initially reported by Icho et al., who isolated the gene for Escherichia coli by complementation of an E.coli mutant defective in the enzyme activity. The first eucaryotic gene encoding CDS was cloned by Wu et al. from Drosophila. Using a molecular genetic screen designed to isolate genes important for the in vivo regulation of phototransduction in Drosophila, a PLC-mediated signal transduction cascade, they isolated and identified the CDS gene as a key enzyme gene in PLC signaling. Since then, the gene for yeast and cDNAs for human have been isolated by computer assisted research. Weeks et al. have shown that the human CDS protein and the CDS proteins from Drosophila, yeast and E. coli have 45%, 21%, and 7% overall match in amino acid sequence, respectively.

In the present investigation, we have cloned a cDNA encoding CDS from rat brain to understand the function and the regulation of CDS in the central nervous system. Furthermore, with the use of the cloned cDNA, we investigated the distribution and the expression during the development of the CDS mRNA in the rat central nervous system, by in situ hybridization and reverse transcription-polymerase chain reaction (RT-PCR).

**MATERIALS AND METHODS**

**Animals**

Timed-pregnant Wistar rats were maintained with access to food and water. Offspring were divided into groups of six rats and studied at postnatal day 1 (P1), P7, P14, P21, P28 and 12 weeks (adult).

**Construction of a rat brain cDNA library**

Total RNA was isolated from the brain of a male Wistar rat (3 weeks old) by the acid guanidium thiocyanate extraction method. Poly(A)+RNA was obtained from total RNA by oligo-dT-latex beads (Takara Co. Ltd.). Five micrograms of poly (A)+RNA were subjected to the synthesis of double-stranded cDNA by a cDNA synthesis kit (Life Technologies, Inc.) using oligo-dT as a primer. The double stranded cDNA was then ligated to EcoRI/NotI adaptor, and the adaptor ends were phosphorylated with a T4 poly nucleotide kinase (Pharmacia). The adaptor-ligated and 5'-phosphorylated cDNA was ligated to lamda ZAP Express pharge arms (Stratagene) before being packaged in vitro by using Gigapack II gold (Stratagene) to generate a cDNA library. The cDNA library contained $5 \times 10^6$ independent clones.

**cDNA cloning and DNA sequence**

Two expressed sequence tags (EST) clones from human placenta (I.M.A.G.E. Clone ID # 133825 and # 135630) were obtained from the I.M.A.G.E. Consortium, through Research Genetics (Huntsville, AL), and the mixture of the two excised inserts was used as probes for a low-stringency hybridization. Approximately $3 \times 10^4$ clones were screened with the mixture of the [32P]-labeled probes. Three pharge clones, designated #1, #2 and #3, with inserts of 3.7 kbp, 2.7 kbp and 2.7 kbp length, were isolated. According to the supplier’s instruction, the pharge clones #1, #2 and #3 were converted to phargemid clones named pCDS1, pCDS2 and pCDS3, respectively. Based on their restriction maps, they could be classified into two groups: one clone (pCDS1) and two clones (pCDS2 and pCDS3). Nucleotide sequences of the insert DNA of pCDS1 and pCDS3 were determined from both ends using a DNA sequencing kit (Perkin-Elmer) on the ABI 377 DNA sequencer (Perkin-Elmer).

**Northern blot analysis**

A multiple-tissue Northern blot was purchased from Clonetech. The blot was probed with the whole insert of pCDS1 as described previously.

**In situ hybridization**

The pCDS103 for cRNA synthesis was prepared as follows: pCDS3 was digested with BamHI and BglII to remove the 1.2 kbp insert, purified by an agarose gel electrophoresis, and religated to form pCDS103 which contains 465 nucleotides corresponding to the positions 1090-1555 of the CDS cDNA. Digoxigenin (DIG)-labeled antisense cRNA for CDS was synthesized by the T3 RNA polymerase (Ambion) reaction with HindIII digested pCDS103 as the template using a DIG RNA labeling kit (Boehringer Mannheim) according to the supplier’s instruction. The sense RNA was synthesized in a similar manner using T7 polymerase and Sall digested pCDS103. Wistar rats were decapitated, and the brains were rapidly excised, embedded in Tissue-Tec OCT compound (Miles Inc.), frozen, and stored at $-80^\circ$C until use. In situ hybridization was performed as described previously.

**RT-PCR**

Total RNA was extracted from each stage of the rat olfactory bulb, hippocampus, cerebellum and spinal
cord by a single-step method using ISOGEN (Nippon Gene, Toyama) developed by Chomczynski and Sacchi\(15\). Total RNA (0.1\(\mu\)g) was reverse-transcribed and subjected to PCR amplification using a one-step RT-PCR kit (Toyobo) according to the manufacturer’s protocol except that the reaction volume was reduced to 25\(\mu\)l. The primers used for amplification of CDS cDNA were sense 5’-GGCTAGAAGCTCAGTATGTAG and antisense 5’-AAATGCTAGGTTGCTAGTAGA corresponding to the base sequences 1472-1491 and 1836-1855. The reverse transcription was conducted at 60°C for 30 min, followed by incubation at 94°C for 2 min. PCR amplification was performed at 94°C for 1 min and at 62°C for 1.5 min for 28 cycles, followed by a final extension at 60°C for 7 min. The primers used for amplification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) cDNA were sense 5’-TCCACCACCTGTGTGTA and antisense 5’-ACACAGTCCATGCGCATC. The reverse transcription was conducted at 60°C for 30 min, followed by incubation at 94°C for 2 min. PCR amplification was performed at 94°C for 1 min and at 65°C for 1.5 min for 23 cycles, followed by a final extension at 60°C for 7 min. PCR products (5 \(\mu\)l) were subjected to electrophoresis on a 10% agarose gel stained with ethidium bromide, visualized under UV light, and photographed through an SL 39 u.v. filter (Mamiya, Tokyo) onto film (Polaroid 667, Polaroid, Tokyo). The correct size of the PCR product was determined by using a standard DNA marker. The photograph was scanned with an image scanner (GT8500, Epson, Tokyo) and analyzed with a personal computer (Power Macintosh 7500/100, Apple Computer Inc., Cupertino, CA). The intensity of each band visualized as an ethidium bromide fluorescence was measured using NIH Image free software (Version 1.44b, Dr. Wayne Rasband, NIH, Bethesda, MD).

**RESULTS AND DISCUSSION**

**Cloning of rat CDS cDNA**

A homology search of the GenBank database using the *Drosophila* CDS protein sequence identified two human ESTs. Using a mixture of the cDNA inserts as probes, three clones were isolated from the rat brain...
cDNA library by low-stringency hybridization. Nucleotide sequences of the two clones were determined. As shown in Fig. 1, the sequences overlapped, indicating that they were derived from an identical transcript. Fig. 1 shows that the cDNA has a single open reading frame of 461 amino acids with a predicted molecular weight of 52,927 Da. The predicted protein contains the same number of amino acids as that of the human CDS and is 95% identical (data not shown) to the human CDS. Therefore, we concluded that the predicted protein encoded by the cDNA isolated in this study is the rat homologue of the human CDS. During the sequence of the CDS clones we noticed the report by Saito et al., who cloned and sequenced the cDNA encoding CDS from rat brain. The nucleotide sequence of the coding region of their cDNA was essentially the same as that of ours with a minor difference: three Gs at positions 894, 916 and 926 of their clone were missing in our clone. Because of this difference, an undeca peptide VQVSVLCLGARW at amino acid positions 299-309 encoded by their clone was changed to a deca peptide SKYQYFVCVPV at positions 299-308 encoded by our clone. The reason for the difference is not clear. However, the deca peptide sequence region of our rat CDS was conserved completely in human CDS and well in yeast.

Fig. 2. In situ hybridization of CDS mRNA in the adult rat brain. A: coronal sections of the hippocampus hybridized with a DIG-labeled CDS antisense RNA. B: Dentate gyrus (DG). C: high magnification of DG. D: A negative control section of the hippocampus hybridized with a cRNA probe encoding the sense strand. E: high magnification of the cerebellum. M, P, and G designate molecular, Purkinje, and granular cell layers, respectively. F: high magnification of the dorsal root ganglion. Bars = 500 μm in A and D, 200 μm in B and F, 50 μm in C and E.
Northern blot analysis demonstrated that rat CDS mRNA was differentially expressed in various tissues (data not shown). Higher expression was seen in the testis, brain and kidney in this order, but lower expression was seen in the liver. A 4 kb transcript was detected commonly in these four tissues, but a 2.4 kb transcript was detected only in the testis. The transcripts were hardly detected in the heart, lung and skeletal muscle.

In situ hybridization

We examined the precise distribution of CDS mRNA using non-radioisotopic in situ hybridization, a procedure which allows highly resolved detection of positive neurons. Antisense and sense RNA probes labeled with DIG were prepared by in vitro transcription directed by the T3 and T7 promoters. Sections of various regions of rat brain were prepared, and RNA probes were applied and stained. Signals were obtained only with the antisense probe. The sense probe used as the control produced essentially no hybridization signals (Fig.2D). Table 1 summarizes the distribution of CDS mRNA in the central nervous tissue of the adult rat.

Moderate expression of CDS mRNA was seen in the mitral cell layer of the olfactory bulb, and weak expression in the granular cells that lie in clusters near the mitral cells. However the CDS mRNA hybridization signal was lacking in the glomerular layer. CDS mRNA was weakly stained in the piriform cortex. CDS mRNA was expressed moderately and evenly in the neural cell bodies of layer V/VI. In the hippocampus, moderate expression of CDS mRNA was observed in the granular cells of the dentate gyrus, and in the pyramidal cells of the CA 1-4 (Fig.2A, B, and C). In the basal ganglia, neurons in the caudate putamen were not labeled with CDS mRNA probe although most of neurons in the globus pallidus were weakly labeled. CDS mRNA was also weakly stained in the amygdaloid nuclear complex, including the medial, basolateral, and central amygdala.

CDS transcript was not expressed in the epithalamus. In the thalamus, CDS signals were moderately expressed in the ventral part of the nuclei.

In the hypothalamus, weak levels of CDS mRNA were observed in the ventromedial hypothalamic nucleus, lateral hypothalamic nucleus, and supramammillary nucleus.

Moderate to weak hybridization signals of CDS mRNA were seen in the midbrain, pons, and medulla. In the cerebellum, the CDS mRNA hybridization signal was moderate in the Purkinje cells (Fig.2E) and weak in the granule cells. Signals were not detected in the molecular layer.

We examined the sections of the spinal cord at the lumbar level. CDS mRNA was predominantly localized in the gray matter which included the cell bodies of the motoneuron. Moderate hybridization signals were seen in the ventral horn and dorsal root ganglion (Fig.2F). As in other regions, hybridization was never observed in association with white matter tracks.

Moderate hybridization signals were found homogeneously in the anterior and intermediate lobes of the pituitary.

The expression pattern of CDS mRNA we observed in the present study generally agrees with the results of in situ hybridization study with radioisotope-labeled DNA probes.

Developmental changes in expression

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<th>Dentate gyrus</th>
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In situ hybridization signals obtained with DIG-labeled antisense probe were assessed as moderate (+ +), weak (+), or not detectable (−).
We next studied the developmental changes in the expression of CDS mRNA in the olfactory bulb, cerebellum, hippocampus, and spinal cord. The RT-PCR method was employed to quantitatively determine the CDS mRNA relative to the expression of G3PDH mRNA, which is widely used as an internal standard for gene expression studies.

In the olfactory bulb (Fig.3A), the PCR product was readily detectable at P1. The most dramatic increase was seen between P1 and P7 where a nearly three-fold increase occurred. The peak expression was noted at P14 then gradually declined to the adult stage in which the levels are slightly more intense than at P1.

In the cerebellum (Fig.3B), the level of the PCR product was quite low for newborn (P1). The levels increased during the first to second postnatal weeks, reaching a plateau at P14 and then remained constant until adulthood where a nearly four-fold increase occurred.

In the hippocampus (Fig.3C), the pattern is similar

Fig. 3. CDS gene expression in each region, olfactory bulb (A), cerebellum (B), hippocampus (C), and spinal cord (D) of male rats at different ages during postnatal development and at adulthood. RT-PCR analysis was conducted as described in MATERIALS AND METHODS. The intensities of CDS bands were normalized using the G3PDH PCR band as the internal standard. The PCR band intensities of CDS mRNA in each part of the rat brain on P1-P28 and in adulthood were expressed as relative values taking the value at P1 as 100.
Rat CDP-diacylglycerol synthase

to that of the olfactory bulb; the expression increasing dramatically, and reaching a high level at P7 (4.5-fold that of P1). The levels reached a peak at P21 and then declined slightly.

In the spinal cord, Fig.3D showed a dramatic increase in the expression of CDS mRNA between P1 and P7 (5-fold that of P1). The levels then increased gradually until adulthood.

The common features of these results are dramatic increases in the expression of CDS mRNA during the early stage of postnatal growth, although some differences were seen in the late stage. The elevation of the expression seems to be associated with postnatal maturational events including axonal extension, synaptogenesis and myelination.

ACKNOWLEDGMENT

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