Gene Cloning and Characterization of CDP-diacylglycerol Synthase from Rat Brain*

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Sachiko Saito‡§, Kaoru Goto‡, Akira Tonosaki§, and Hisatake Kondo‡¶

From the ‡Department of Anatomy, Tohoku University School of Medicine, Sendai 980-77 and the §Department of Anatomy, Yamagata University School of Medicine, Yamagata 990-23, Japan

A cDNA encoded a 462-amino acid protein, which showed CDP-diacylglycerol synthase (CDS) activity was cloned for the first time as the vertebrate enzyme molecule from rat brain cDNA library. The deduced molecular mass of this rat CDS was 53 kDa, and putative primary structure included several possible membrane-spanning regions. At the amino acid sequence level, rat CDS shared 55.5%, 31.7%, and 20.9% identity with already known Drosophila, Saccharomyces cerevisiae, and Escherichia coli CDS, respectively. This rat CDS preferred 1-stearoyl-2-arachidonoyl phosphatidic acid as a substrate, and its activity was strongly inhibited by phosphatidyglycerol 4,5-bisphosphate. By immunoblotting analysis of COS cells overexpressed with the epitope-tagged for rat CDS, a 60-kDa band was detected. By epitope-tag immunocytochemistry, the CDS protein was mainly localized in close association with the membrane of the endoplasmic reticulum of the transfected cells. The intense mRNA expression of CDS was localized in the cerebellar Purkinje cells, the pineal body, and the inner segment of photoreceptor cells. Additionally, very intense expression was detected in postmitotic spermatocytes and spermatids.

Phosphoinositide cycle mediates one of the intracellular signal transduction pathways in eukaryotic cells and produces a class of second messengers that are involved in cell growth (1, 2), differentiation (3, 4), the action of hormones and neurotransmitters (5), and sensory perception (6–8). Triggering of tyrosine kinase receptors, initiates the cycle by activating transmitters (5), and sensory perception (6–8). Triggering of class of second messengers that are involved in cell growth (1, 2), differentiation (3, 4), and sensory perception (6–8) is well known, such as the primary second messenger role of cGMP in vertebrate photoreceptors versus that of IP₃ in invertebrate. However, recent investigations have shown unique distribution of phosphoinositide-specific PLC (13–15), protein kinase C (16, 17), and IP₃ receptor (18) in vertebrate rod outer segment, and that light may enhance the activity of PLC (19–22) and protein kinase C (23), and phosphoinositide synthesis (24). In addition, it has been reported that cytoplasmic Ca²⁺ concentration mediates light adaptation in vertebrate photoreceptors (25, 26). Thus it is suggested that in vertebrates as well as invertebrates the phosphoinositide cycle may play a role in the phototransduction signaling. It is therefore possible that CDS is also important in the signal transduction mechanism of vertebrate retina and neural cells.

As a step toward understanding the possible functional significance of this enzyme in vertebrate cellular signaling, the present study was attempted to perform molecular cloning of a CDS molecule from rat brain and to clarify its enzymatic feature and tissue and cell localization. Our result shows that a newly identified CDS prefers arachidonate-containing PA as a substrate, suggesting strongly a role for CDS in the phosphoinositide synthesis. It is also shown that the activity of CDS is inhibited by PIP₂, suggesting that polyphosphoinositides regulate their own synthesis through CDS activity. In addition, we show the detailed localization at mRNA level in the retina and brain, but unexpectedly the highest expression of its mRNA is detected in the testis.

EXPERIMENTAL PROCEDURES

Polymerase Chain Reaction (PCR)—Total RNA was extracted from adult Wistar rat brain by acid guanidinium thiocyanate-phenol-chloroform extraction (27). Poly(A)⁺ RNA was isolated by chromatography on an oligo(dT)-cellulose column. First-strand cDNA was prepared using First-Strand cDNA Synthesis kit (Pharmacia Biotech Inc.). Primers used for PCR were composed of two degenerate oligonucleotides, which were made according to the amino acid sequences of conserved regions between Drosophila CDS (12) and E. coli CDS (28): the

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regions corresponded to the amino acid sequences TW/E/QG/GFG (aa 276–283 for 5’ primer) and IPHGHHG/M/L (aa 391–398 for 3’ primer) (amino acid numbers represent those of Drophila CDS). The sequences for the primers were designed according to the mammalian cDNA (18). Sequence analysis on both strands as described above.

To analyze the sensitivity of CDS activity to -glycero-3-phosphate (C18:0/C20:4-sn and 1-stearoyl-2-arachidonoyl-PC) as long chain PAs. To analyze the sensitivity of CDS activity to -glycero-3-phosphate (C18:0/C20:4-sn and 1-stearoyl-2-arachidonoyl-PC) as long chain PAs. To analyze the sensitivity of CDS activity to -glycero-3-phosphate (C18:0/C20:4-sn and 1-stearoyl-2-arachidonoyl-PC) as long chain PAs.

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C18:1-PA, and PA from egg yolk lecithin were 102, 114, and 138 μM, respectively; and for the apparent 
\( V_{\text{max}} \) were 268, 259, and 198 pmol/min/mg total protein, respectively. Values for apparent 
\( V_{\text{max}}/K_m \) (specificity constant) calculated from Eadie-Hofstee plots were 2.51, 2.28, and 1.48, respectively. When the 
utilization of CTP and dCTP for the activation of phospholipid 
intermediates was compared, dCTP was incorporated into 
phospholipid precursors at almost the same rate as CTP. In the 
analysis of inhibitory effects of PI, PIP, and PIP2 on the CDS 
activity of the present molecule, the activity decreased by 50% 
at 2 mol% PIP2 and by 80% at 10 mol% and more (Fig. 4), under 
the assay condition at 1000 μM C18:0/C20:4-PA (the present 
molecule showed the maximum activity at 200–1000 μM C18: 
0/C20:4-PA). In contrast, the activity decreased by at most 40% 
at 5–20 mol% PIP. Addition of PI at various concentrations 
from 2 to 20 mol% produced little effects on CDS activity. 
Similar results were obtained in the experiments for C18:0/ 
C20:4-PA and di-C18:1-PA at 100 or 500 μM (data not shown).

In the immunoblotting of epitope (FLAG)-tagged CDS with 
the antibody against the FLAG tag, a single immunoreaction 
band was observed at size of 60 kDa (Fig. 5).

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cell population and they were randomly dispersed in each culture dish. The immunoreactivity was localized widely throughout the cells in forms of delicate networks composed of fine dots with a tendency of condensation in one pole of juxtanuclear cytoplasm, and they were also deposited along the nuclear rim. In immunoelectron microscopy (Fig. 6), the immunoreactivity was detected along the membranes of endoplasmic reticulum, vesicles and vacuoles, and nuclear envelopes. No immunoreactivity was detected in any cells when the transfection was made of the cDNA without the FLAG tag.

In Northern blot analysis (Fig. 7) of adult rat on postnatal day 49, the hybridization bands were detected very strongly in testis at sizes of 3.5 and 2.2 kb, suggesting a possibility of alternative splicing. The hybridization band was strongly detected in eyeball and brain at a size of 3.5 kb. Among other tissues, a weak band of 3.5 kb was detected in kidney, small intestine, and placenta, whereas a faint band of 3.5 kb was seen in thymus and lung after long exposure. Any hybridization bands could not be detected in heart, liver, and ovary.

By in situ hybridization histochemical analysis of the adult brain (Fig. 8), the most intense expression signals were detected in the cerebellar Purkinje cells and intense expression was found...
in the pineal body. Moderate to low expression was seen in layers II–VI of the cerebral cortex, in the hippocampal pyramidal cell layer and subiculum, and the olfactory mitral cells. Low expression was seen in almost all neurons in the fore-, mid- and hind-brains with much lower expression in the caudate putamen. Low expression was also seen in the choroid plexuses. No significant expression was detected in the white matter including the corpus callosum and cerebellar medulla or in the glia limitans. In the retina, positive expression signals were confined to the inner segment of the photoreceptor cells (Fig. 9).

In the adult testis, the hybridization signals were densely deposited in most of the adluminal compartment of the seminiferous tubule composed of postmitotic spermatocytes and spermatids, while no significant signals were detected in the apical compartment composed of spermatozoa or in the basal compartment composed of proliferating spermatogonia. No significant signals were found in the interstitial cell clusters (Fig. 10).

In the control experiment in which several random sections of brain, retina, and testis were hybridized with the labeled oligonucleotide probe in the presence of 100-fold excess amount of the unlabeled probe, no significant expression signals were detected in any regions of the tissue sections.

DISCUSSION

We report here for the first time the molecular cloning of vertebrate CDS and clarify its primary structure, enzymatic features, and localization in detail. The primary structure of this rat CDS shared 55.5%, 31.7%, and 20.9% identity to that of Drosophila (12), S. cerevisiae (36), and E. coli (28), respectively (Fig. 2a). The high hydrophobicity and the inclusion of several putative membrane-spanning regions in the primary structure of the present molecule and the localization associated with the endoplasmic reticulum and nuclear envelopes are well consistent with available biochemical data of the mammalian CDS so far reported (34–41). The deduced molecular size of 53 kDa is smaller than the size of 60 kDa detected by epitope-tagged immunoblotting. This discrepancy may be ascribed to the post-translational modification of CDS in vivo. From the sequence, it is clear that there are several potential serine/threonine phosphorylation sites, suggesting the possibility that phosphorylation by serine/threonine kinases may be involved in regulation of this enzyme activity.

The present study shows the substrate specificity of rat CDS toward 1-stearoyl-2-arachidonoyl PA (C18:0/C20:4-PA) among several PAs (Fig. 3). In mammalian cells, CDP-DAG is used to produce phosphatidylinositol, phosphatidylglycerol, and cardiolipin, and CDP-DAG is therefore an important regulatory branching point in the phospholipid metabolism. Since the PI predominantly consist of the 1-stearoyl-2-arachidonoyl species...
the present substrate specificity of rat CDS suggests strongly that this enzyme molecule selectively participates in the phosphoinositide cycle, but not in the synthesis of phosphatidyglycerol and cardiolipin. The present study also clarifies the marked inhibition of rat CDS activity in vitro by PIP₂ (Fig. 4), a presumed end product of the phosphoinositide cycle. This represents another new example of the potential for acidic phospholipids including phosphoinositides to modulate the in vitro activities of several membrane enzymes related to the signal transduction. PI-4-phosphate 5-kinase appears to be inhibited by PIP₂ and be activated by PA (43–45), casein kinase I appears to be inhibited by PIP₂ (46), phosphoinositide-specific PLC-γ₁ appears to be activated by PA (47), and several protein kinase C isoform are activated by PIP₂ (phosphatidylinositol 3,4-bisphosphate as well as phosphatidylinositol 4,5-bisphosphate) and phosphatidylinositol 3,4,5-trisphosphate (48–50). This PIP₂-induced enzymatic inhibition and the substrate specificity of rat CDS suggest the presence of a mechanism for PIP₂ to regulate its own synthesis through the phosphoinositide cycle by feedback.

The PIP₂-induced inhibition has recently been demonstrated to occur on arachidonoyl-DAG kinase, which catalyzes the synthesis of PA from sn-1-acyl-2-arachidonoyl-DAG selectively by phosphorylation (51). Considering the fact that DAG kinase and CDS are sequentially involved in the phosphoinositide resynthesis, the finding that both arachidonoyl-specific DAG and CDS are inhibited by PIP₂ strongly suggests that PIP₂ is synthesized on demand as opposed to being stored, and the conversion process from DAG to CDP-DAG through PA, the initial steps of resynthesis of phosphoinositides, is tightly regulated in the phosphoinositide-mediated signaling cascades. In addition, similar to the CDS molecule, the arachidonoyl-DAG kinase has been shown to be expressed also highly in the testis as well as the brain and to be an integral membrane protein (52). It is thus suggested that these two arachidonoyl-specific enzyme molecules are located in the same subcellular membranes and are regulated by the lipid microenvironment within the membranes in a paired fashion in the two consecutive reactions of the phosphoinositide cycle. The detailed cellular...
and subcellular localization of the arachidonoyl-DAG kinase in the testis as well as the brain and retina, and its comparison with that of this CDS may be informative in this regard. The functional significance of the most intensive expression of rat CDS mRNA in tests, especially in postmitotic spermatocytes and spermatids remains to be elucidated.

The localization of this CDS mRNA in the inner segment of rat photoreceptor cells (Fig. 9) corresponds well to the presence of the Drosohila homologue in the photoreceptor cells, suggesting some similar functional significance between the two homologous molecules of far remote animal species. As cited in the Introduction, it has clearly been demonstrated that phosphoinositide-specific PLC isoforms cloned from mammalian homologous organ to the retina, is in favor of this possibility. The functional significance of CDS in the neuronal signal transduction of the functional significance of this enzyme in the phosphoinositide-related signal pathway.

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Fig. 10. Bright field light micrograph (a) and dark field light micrograph (b) of rat CDS mRNA expression in the seminiferous tubule of adult rat testis by in situ hybridization. Note intense hybridization signals in the zone of postmitotic spermatocytes (pSc) and spermatids (Sg). Sg, spermatogonia; Sz, spermatozoan; I, interstitium. (Hematoxylin stain, 25 μm thickness, autoradiographed for 30 days; bars = 200 μm.)
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