Nuclear Localization of Enzymatically Active Green Fluorescent Protein-CTP:Phosphocholine Cytidyltransferase \( \alpha \) Fusion Protein Is Independent of Cell Cycle Conditions and Cell Types*

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To address the recent controversy about the subcellular localization of CTP:phosphocholine cytidyltransferase \( \alpha \) (CT\( \alpha \)), this study was designed to visualize green fluorescent protein (GFP)–CT\( \alpha \) fusion proteins directly and continuously under different conditions of cell cycling and in various cell lines. The GFP–CT\( \alpha \) fusion proteins were enzymatically active and capable of rescuing mutant cells with a temperature-sensitive CT. The expressed GFP–CT\( \alpha \) fusion protein was localized to the nucleus in all cell lines and required the N-terminal nuclear targeting sequence. Serum depletion/replenishment did not cause shuttling of CT\( \alpha \) between the nucleus and cytoplasm. Moreover, the subcellular localization of CT\( \alpha \) was examined continuously through all stages of the cell cycle in synchronized cells. No shuttling of CT\( \alpha \) between the nucleus and cytoplasm was observed at any stage of the cell cycle. Stimulation of cells with oleate had no effect on the localization of CT\( \alpha \). The GFP–CT\( \alpha \) lacking the nuclear targeting sequence stayed exclusively in the cytoplasm. Regardless of their localization, the GFP–CT\( \alpha \) fusion proteins were equally active for phosphatidylcholine synthesis and mutant rescue. We conclude that the nuclear localization of CT\( \alpha \) is a biological event independent of cell cycle in most mammalian cells and is unrelated to activation of phosphatidylcholine synthesis.

CTP:phosphocholine cytidyltransferase (CT\( \alpha \)) is the regulatory enzyme of the CDP-choline pathway for de novo synthesis of PC (1). CT catalyzes the second reaction between choline kinase and cholinephosphotransferase. The CDP-choline pathway is present in all mammalian cells and is essential for cell survival (2). Complete inactivation of CT by a temperature-sensitive mutation leads to apoptosis (3). CT\( \alpha \) was first purified by Weinhold and co-workers in 1986 (4), and its cDNA was first cloned from rat liver by Cornell and co-workers in 1990 (5). PC synthesis is nearly normal even when over 90% of CT is inactivated (2), suggesting that most cellular CT remains inactive for PC synthesis. However, nearly all cellular CT\( \alpha \) has been found localized in the nucleus (6) although the major site of PC synthesis was found associated with the endoplasmic reticulum (7–9). Because of this physical separation of CT\( \alpha \) localization and PC synthesis, the precise localization of CT\( \alpha \) and universality of CT\( \alpha \) localization in the nucleus have become interesting topics for investigation.

Since the nuclear localization of CT\( \alpha \) was first reported by Kent and co-workers (6), the subcellular localization of CT\( \alpha \) has become increasingly controversial. A study by Houweling et. al (10) demonstrated that significant staining of CT was detected in both the cytoplasm and nucleus of primary rat hepatocytes and of rat liver thin slices. Adding to this complexity, two more isoforms of CT (CT\( \beta \)1 and \( \beta \)2) without the N-terminal nuclear targeting signal were found in animal tissues, including liver, and were present in the cytoplasm (11). It is not known how the participation of CT\( \alpha \) in the CDP-choline pathway is regulated, because the amino acid sequences responsible for several previously suspected mechanisms of CT\( \alpha \) activation can be deleted without affecting its involvement in the CDP-choline pathway (12–14). In a recent study by Cornell and co-workers (15), a novel mechanism for CT\( \alpha \) activation was proposed. Based on the observations that activation of PC synthesis during the G\( \text{0} \) to G\( \text{1} \) transition was accompanied by a translocation of CT\( \alpha \) from the nucleus to the cytoplasm in cultured IIC9 cells, it was proposed that this translocation was a mechanism for CT\( \alpha \) activation. Yet, this proposed mechanism of translocation from the nucleus to the cytoplasm might not be necessary if two other isoforms of CT are already present in the cytoplasm. Therefore, it inevitably becomes important to determine the specific localization of CT\( \alpha \) in other mammalian cells and to verify whether the observation of such a cell cycle-dependent translocation between the nucleus and the cytoplasm could be extended to other mammalian cells.

In this study, we devised a new strategy of monitoring the subcellular localization of CT\( \alpha \) directly and continuously in live mammalian cells at various stages of the cell cycle. This study was designed to address the following questions. 1) Is the nuclear localization of CT\( \alpha \) a universal event and present in other mammalian cells? The subcellular localization of CT\( \alpha \) was previously investigated in four types of mammalian cells: Chinese hamster ovary cells (CHO-K1 and MT58) (6, 12, 16), human hepatocarcinoma cells (HepG2) (6), mouse fibroblast cells (NIH3T3, L-cells, and IIC9) (6), and primary rat liver cells (6, 10). Our current study extended observations into six additional cell lines; the study found no evidence of translocation but did find constitutive localization of CT\( \alpha \) in the nucleus of all cell lines tested. 2) Is the CT\( \alpha \) translocation between the nucleus and the cytoplasm in IIC9 cells during serum depletion/replenishment (15) also present in other mammalian cells? We examined CT\( \alpha \) localization in Chinese hamster ovary cells un-
under similar conditions and found no evidence of translocation. 3) Is the subcellular localization of Ctα changed during the synchronized movement of cells through all stages of the cell cycle? Because the subcellular localization of Ctα in continuous stages of the cell cycle has never been addressed before, we examined the localization of Ctα in synchronized cells at different stages of the cell cycle in Chinese hamster ovary cells. Additionally, the use of GFP-Ctα fusion proteins for direct visualization in cells circumvented specificity problems inherent with indirect immunofluorescence detection. In this report, we describe the findings of this study.

EXPERIMENTAL PROCEDURES

Materials—Chinese hamster ovary cells (K1 and MT58), rat hepatoma cells (MA-RH 7777), rat embryo fibroblasts, sheep choroid plexus cells, baby hamster kidney cells, and human hepatoblastoma cells (HeP2) and human mammary gland (MCF-10A) and human normal lung (MRC-9) cell lines were from American Type Culture Collection. Dulbecco’s modified Eagle’s medium (DMEM), Eagle’s minimal essential medium, F-12 nutrient mixture (F12), DMEM/F12, and fetal bovine serum (FBS) were from Life Technologies, Inc. [methyl-3H]choline chloride was from PerkinElmer Life Sciences. Anti-full-length GFP antibody was from Santa Cruz Biotechnology. All other chemicals and reagents were from Fischer.

Cell Culture—Rat primary hepatocytes were obtained by collagenase perfusion (17). Hepatocytes were cultured on collagen-coated culture dishes and incubated in DMEM with 10% FBS, 10 μg/ml insulin, and 10 mM Hepes buffer at 37 °C overnight prior to transfection. K1 and MT58 cells were cultured in F12 medium with 10% FBS at 33 °C. Hep2 and sheep choroid plexus cells were cultured in Eagle’s minimal essential medium with 10% FBS at 37 °C. MCF-10A cells were cultured in DMEM/F12 with 10% FBS at 37 °C. Other cells were cultured in DMEM with 10% FBS at 37 °C.

CT-GFP Fusion Constructs—The cDNA for Aequorea victoria GFP (Life Technologies, Inc.) was cloned into the mammalian expression vector pcDNA3. For construction of GFP-Ctα-pCDNA3, the GFP-specific 5′ and 3′ primers with EcoRI sequences were used to generate a polymerase chain reaction fragment of GFP with removal of the start and stop codons and addition of an EcoN1 restriction site to each end of the GFP coding region (EcoN1-GFP-EcoN1). Full-length cDNA for rat liver Ctα in the mammalian expression vector pCDNA3 (CT-pCDNA3) was digested with EcoN1, which cuts at a unique site between residues 58 and 59 of Ctα, and treated with calf intestine alkaline phosphatase. The treated Ctα-pCDNA1 was ligated to the EcoN1-GFP-EcoN1 fragment, and correct orientation of the GFP insertion was confirmed with restriction mapping. This construction places GFP behind the nuclear targeting region (residues 8–28) (12) of Ctα, but in front of the catalytic domain (residues 72–236) (19) of Ctα.

RESULTS

Localization of Ctα in the Nucleus

Fluorescence Microscopy—Cells transfected with GFP-pCDNA3, GFP-Ctα-pCDNA3, and GFP-Ctα-pCDNA3 plasmids were observed after 48 h under a Zeiss Axioplan-2 epifluorescence microscope equipped with a fluorescence filter. Digital images of cells were recorded using a Spot camera.

Flow Cytometry—Cells were prepared for flow cytometric analysis by fixing cells (5 × 105) in ethanol and suspending cells in 50 μl/mg propidium iodide, 0.6% Nonidet P40, and 37 μg/ml RNase. The stained cells were analyzed on a Coulter XL flow cytometer, which excites the cells at 488 nm and measures the red fluorescence per cell, which can be equated to the DNA content per nucleus.

Fluorescence Microscopy—Cells transfected with GFP-pCDNA3, GFP-Ctα-pCDNA3, and GFP-Ctα-N-pCDNA3 plasmids were observed after 48 h under a Zeiss Axioplan-2 epifluorescence microscope equipped with a fluorescence filter. Digital images of cells were recorded using a Spot camera.

Synchronization of MT58 GFP-Ctα Cells—Cells (7 × 105) were plated into a 150-mm culture dish in Ham’s F-12 medium with 10% fetal calf serum and incubated for 1 h at 33 °C. The cell monolayer was washed and then incubated in Ham’s F-12 medium with 0.25 μg/ml nucodazole for 16 h at 33 °C. The cells blocked at the G2/M border were either unattached or lightly attached to the dish. These cells were collected by gentle washing with the culture medium, centrifuged, and resuspended in medium. 3 × 105 cells were plated in 35-mm culture dishes and incubated at 33 °C for 1 h. The medium was replaced with fresh medium containing 2 mM hydroxyurea and further incubated for 9 h. The medium was replaced with fresh F12 and incubated for various time periods, after which the cells were visualized by microscopy or harvested for flow cytometric analysis.

Flow Cytometry—Cells were prepared for flow cytometric analysis by fixing cells (5 × 105) in ethanol and suspending cells in 50 μl/mg propidium iodide, 0.6% Nonidet P40, and 37 μg/ml RNase. The stained cells were analyzed on a Coulter XL flow cytometer, which excites the cells at 488 nm and measures the red fluorescence per cell, which can be equated to the DNA content per nucleus.

RESULTS

The Expressed Fusion Proteins Were Intact—We made three constructs for transfections as diagrammed in Fig. 1. In the first construct, expression of GFP alone was used as a control for determination of transfection efficiency and for subcellular distribution of a protein without a specific targeting signal. GFP was distributed evenly throughout cellular organelles. In the second construct, the GFP coding region was inserted in-frame between the nuclear targeting signal and the last of Ctα (between residues 58 and 59). This construct was designated GFP-Ctα. In the third construct, the nuclear targeting sequence of Ctα was removed from the second construct; this third construct was designated GFP-Ctα-N. The expression plasmids were introduced into the cultured MT58 and K1 cells to verify whether the fusion proteins were intact. Transfections were...
achieved by calcium phosphate precipitation, and the stably transfected cells with green fluorescence were selected in the presence of 500 μg/ml G418. Specific localization of the fusion proteins depended completely on the fusion proteins remaining intact after being expressed. Otherwise the localization of green fluorescence would not represent the localization of the fusion protein but rather degradation fragments of the fusion protein. To verify the expression of the constructed fusion proteins, MT58 cells transfected with the plasmids were probed with specific antibodies against GFP by Western blot analysis. Fig. 2 depicts the presence of fusion proteins in the stably transfected cells. The GFP-CT construct produced a fusion protein with an apparent molecular mass (70 kDa) of the predicted fusion protein between CT (43 kDa) and GFP (27 kDa). The GFP-CTDN construct produced a smaller fusion product corresponding to the removal of the 40 amino acid residues of the CTα nuclear targeting signal. There are two bands in MT58 cells that reacted nonspecifically to the GFP antibody and were not related to GFP. The control GFP construct produced a large amount of GFP protein at the predicted molecular mass. Because the efficiency of expressing GFP alone is much higher than that of larger fusion constructs, we had to reduce the amount of total protein loaded on the gel to 1/10 of the other lanes to achieve comparable exposures. Therefore, the nonspecific bands in MT58 cells did not show up in this lane. Nevertheless, both fusion proteins remained intact in the stably transfected cells, suggesting that the localization of green fluorescence represents the localization of the intact fusion protein.

CTα-GFP Fusion Proteins Were Enzymatically Active—We then determined whether the fusion proteins were enzymatically active for PC synthesis. The stably transfected MT58 cells were shifted to 40 °C to inactivate the endogenous CT activity. Cells were then labeled with [3H]-choline for 24 h, the organic phase of the labeled cells was extracted, and radioactive counts in each sample were determined. The only way for water-soluble [3H]-choline to get into the organic phase was to complete all three steps of the CDP-choline pathway. In a separate experiment, we determined that PC accounted for 96% of the radioactivity in the organic phase. Thus, the incorporation of [3H]-choline into the organic phase was a direct representation of the activity of the CDP-choline pathway and CT activity in MT58 cells at 40 °C. Results of this experiment showed that both fusion constructs of GFP-CTα were enzymatically active (Fig. 3). We also determined by counting cells after incubation at 40 °C that the fusion proteins were capable of rescuing MT58 cells at the non-permissive temperature. The results showed that both fusion proteins, but not GFP alone, restored the growth of MT58 cells at 40 °C, with generation times similar to that of CT expression in MT58 cells (data not shown).

In summary, the GFP-CTα fusion proteins with or without the nuclear targeting sequence behaved indistinguishably from CTα alone in terms of enzyme activity and ability to rescue MT58 cells at 40 °C. Thus, the localization of the fusion proteins should represent the localization of CTα very closely. These constructs were then expressed in cells from several selected origins to determine directly the localization of the fusion proteins in live cells. This design minimized the effects of nonspecificity often seen with antibody staining and cell fixation and represented cellular localization closer to physiological conditions.

Endogenous CT Did Not Affect the Localization of CT Fusion Proteins—After confirming that the fusion protein did retain the expected enzymatic activity and cellular function, we monitored the transfected MT58 cells by fluorescence microscopy. The main purpose of this design was to further confirm that the fusion constructs would behave similarly to the observation of CTα nuclear localization made originally by Kent and co-workers (12). A similar nuclear localization in MT58 cells would validate that the GFP-CTα fusion construct retains critical determinants for nuclear targeting and that its localization in other cell types is a close reflection of native CTα in the physiological state. GFP-CT fusion proteins were indeed localized exclusively to the nucleus of MT58 cells (Fig. 4A) at both permissive and nonpermissive temperatures. Upon removal of the N-terminal nuclear targeting sequence, GFP-CTDN revealed a clear pattern of nuclear exclusion (Fig. 4A). These results indicated that the fusion constructs displayed patterns of subcellular localization identical to that of native CTα reported previously. Because all cell lines included in our intended survey contain endogenous CT, it was important to determine whether the nuclear localization of overexpressed fusion protein is affected by endogenous CT. We expressed the fusion constructs in K1 cells in which the level of endogenous CT is at least 20-fold higher than that of MT58 at the permissive temperature. Fig. 4B demonstrates that the patterns of the fusion proteins in K1 cells were identical to those of MT58 cells (Fig. 4A) at various levels of expression. This result suggests that the mechanism for nuclear retention is not saturable and that the localization of the expressed fusion protein was not affected by the endogenous CT.

Cell Line-independent Localization of CT in the Nucleus—One of the advantages of using GFP-CT fusion was that the localization could be visualized directly in live cells. This visualization required no prior selection of the transfected cells.
Primary hepatocytes are quiescent cells. However, GFP-z-CT fusion protein provided an opportunity for the nuclear or non-nuclear localization of the overexpressed protein to be directly detected in the transfected primary cells. Forty-eight h after transfection, GFP-z-CT fusion protein was detected exclusively in the nucleus (Fig. 4D). The nuclear localization of GFP-CT fusion protein was seen in all transfected hepatocytes. In contrast, GFP-CTDN fusion protein was exclusively localized to the cytoplasm of all transfected hepatocytes. This suggested that the nuclear localization of GFP-CT fusion protein in hepatocytes was also directed by the N-terminal nuclear targeting sequence of CTα. A similar nuclear localization of GFP-CT fusion protein was also detected in a wide range of cell lines from different species and tissues. These cell lines included rat hepatoma cells (RH777, Fig. 4C), baby hamster kidney cells (Fig. 4E), human hepatoma cells (HepG2, Fig. 4F), sheep choroid plexus (Fig. 4G), rat embryo fibroblast (Fig. 4H), human lung cells (MRC9, Fig. 4I), and human mammary gland (MCF-10A, Fig. 4J). The exclusively cytoplasmic localization of GFP-CTDN fusion protein was observed in all cell lines except rat embryo fibroblast, in which the fusion protein was evenly distributed throughout the cells.

Serum Depletion/Replenishment Caused neither Translocation of CT nor Synchronized Entry of Cells to G1 or S Phase—The cell cycle-associated regulation of PC synthesis (23) and the recent finding of CT translocation from the nucleus to the cytoplasm (15) raised an intriguing possibility that CT may translocate across the nuclear membrane in a cell-cycle dependent manner. Our experimental design of using GFP-CTα fusion constructs allowed us to monitor any potential movement of CT during the cell cycle. To determine whether there was a translocation of GFP-CT fusion protein from the nucleus

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**Fig. 4. Localization of CT-GFP constructs in mammalian cell lines.** Cells were transiently transfected with GFP, GFP-CT, and GFP-CTDN plasmids by calcium phosphate precipitation (except for A, in which MT58 cells were stably transfected as described under "Materials and Methods") and incubated at 33 °C (A and B) or 37 °C (C–J). After 48 h, cells were observed with a fluorescent microscope equipped with UV excitation filters, and digital images of cells were recorded. A, MT58; B, CHO-K1; C, McArdle-RH777; D, rat hepatocytes; E, hamster kidney cells; F, human hepatoma cells (HepG2); G, sheep choroid plexus; H, rat embryo fibroblast; I, human lung cells (MRC-9); and J, human mammary gland (MCF-10A). The left panels show cells in phase contrast, and the right panels show cells of the same field under fluorescence.
to the cytoplasm during the G₂ to G₁ transition, we repeated the serum deprivation/replenishment conditions similar to those described by Cornell and co-workers (15). The stably transfected MT58 cells with GFP-CT fusion plasmid were deprived of serum for 36 h at the permissive temperature. Upon replenishment with 10% serum, the localization of GFP-CT fusion protein was examined with a fluorescence microscope continuously at 2-hour intervals. At all time points, GFP-CT fusion protein was detected exclusively in the nucleus (Fig. 5C). Because serum deprivation/replenishment has been used traditionally to prepare cells for synchronized entry into the G₁ phase of the cell cycle, we examined the populations of cells in each phase of the cell cycle by propidium iodide staining and flow cytometric analysis. Cells stayed at the G₂/G₁ position induced by serum depletion and did not move synchronously into S phase upon serum replenishment (Fig. 5D), suggesting that reentry of G₁ cells into the cell cycle was not synchronized by serum replenishment.

**Nuclear Localization of CT in the Synchronized Population of Cycling Cells**—The failure of serum depletion/replenishment to synchronize cells prompted us to examine the localization of GFP-CT fusion protein in cells synchronized at different phases of the cell cycle by a different method. To achieve a highly synchronized movement of cells through the cell cycle, we used a combination of nocodazole and hydroxyurea treatments, which are capable of synchronizing cells at the G₂/M boundary (25) and the late G₁/S boundary (24), respectively. The cycling cells were collected by first blocking cells at the G₂/M boundary with nocodazole, a specific and reversible inhibitor of mitotic spindle assembly (26). The blocked cells were easily collected because mitotic cells were loosely attached to the culture surface. These cells were then plated onto culture dishes, and the entry into S phase was blocked by 1 mM hydroxyurea, a specific and reversible inhibitor of DNA polymerase (24). In the presence of hydroxyurea, all cells were at the border of late G₁/S (Fig. 5A, top panel). Removal of hydroxyurea triggered the synchronous entry of the cells into the cell cycle at S phase. The combination of nocodazole and hydroxyurea provided very synchronous cell populations in all four major phases of the cell cycle (Fig. 5B). A cell cycle analysis program (ModFit) determined that each phase contained over 70% synchrony (data not shown). Upon continuous examination, GFP-CT fusion protein was localized to the nucleus in all the cells of all phases of the cell cycle (Fig. 5A).

**DISCUSSION**

The current study clearly demonstrates that CTα localization in the nucleus is apparently a cell type- and cell cycle-independent event for mammalian cells. We have also demonstrated for the first time, without the influence of other isoforms of CT, that localization of CTα in primary hepatocytes is also nuclear. It is also the first localization of CTα during all phases of the cell cycle of mammalian cells. This nuclear localization was dependent solely on the permanent N-terminal nuclear targeting sequence of CTα. The mechanism of CTα nuclear targeting seemed unsaturated even in the presence of a high level of exogenous CTα. It also was clear that CTα stayed in the nucleus during all stages of the cell cycle except M phase, when the nuclear membrane is completely disintegrated (27). The complete disintegration of the nuclear membrane would allow a protein of any size without a specific targeting mechanism to be evenly distributed in the cells. The N-terminal nuclear targeting sequence-dependent nuclear localization of CTα also excluded the possibility that CT moved through the nuclear pores passively. The nuclear localization of CTα was apparently universal to all the cell types tested in the current study, because CTα was uniformly nuclear even in the asynchronously synchronized populations of cells. The subcellular localization of CT seems to have no apparent effect on the synthesis of PC. More importantly, the recently reported translocation of CT between the nucleus and the cytoplasm was not observed in any cell type or at any stages of the cell cycle in our current study. We propose two possible explanations for the difference between our current report and the previous report from Cornell and co-workers (15). 1) The serum-induced translocation of CT is an event specific only to IIC9 cells and not a universal event in mammalian cells. 2) The indirect detection of CT in IIC9 cells using antibodies was not specific to CTα.

A major difference between our current findings and previous findings was the CT localization in primary hepatocytes. The study by Houweling et al. (10) demonstrated that significant staining of CT was detected in both the cytoplasm and the nucleus of primary rat hepatocytes. According to the recent findings of CT isoforms (11, 28), we offer an explanation for why CT was detected in both the nucleus and the cytoplasm in primary hepatocytes. The antibody used in the previous studies was raised against a conserved region of CT (16DFV8HD-DIPISSAG). This conserved region is also shared by other isoforms of CT (CTβ1 and CT) that are known to be present.
specifically in the cytoplasm. Therefore, the detection of CT using this antibody was not specific to CTα, and this antibody is capable of recognizing cytoplasmic isoforms of CT, resulting in the detection of both nuclear and cytoplasmic CT.

Another difference between our current study and previous studies is that treatment of cells with oleate did not change the subcellular localization of CTα fusion protein (data not shown). Addition of oleate to HeLa cells has been shown to induce translocation to the nuclear membrane (29). Currently, it is not clear if the failure of GFP-CTα fusion protein to bind nuclear membranes was affected by the fusion construction with GFP. Nevertheless, the failure of GFP-CTα fusion protein to bind to nuclear membranes upon oleate stimulation had no effect on its ability to synthesize PC and rescue MT58 cells at the non-permissive temperature.

Previous constructs of CTα lacking the N-terminal nuclear targeting sequence resulted in CTα localization in both the nucleus and the cytoplasm (12). Our protein fusion design of GFP-CTαDN was the first active CTα construct localized exclusively in the cytoplasm. Such an unexpected specificity for the cytoplasm allowed us to determine whether the nuclear or cytoplasmic localization of CTα would have different impacts on the ability of CTα to synthesize PC and rescue mutant cells lacking CT. The answer was clear that both nuclear CT and cytoplasmic CT were equally effective for PC synthesis and mutant rescue. This observation is consistent with the fact that the molecular weight of CDP-choline is well below the molecular weight cut-off of the nuclear membrane pores. Therefore, the movement of CDP-choline across the nuclear membrane should not be restricted. No matter where CDP-choline is synthesized, it is readily available for PC synthesis. It is not clear why certain cells may have CT present both in the nucleus and in the cytoplasm. The presence of isoforms of CT in the cytoplasmic compartments has diminished the possibility that CTα is required to translocate from the nucleus to the cytoplasmic compartments for PC synthesis. As previously mentioned, the endoplasmic reticulum is a primary site for PC synthesis. PC synthesis via the CDP-choline pathway has been observed to have the channeling effect (30), in which PC can only be labeled by the initial substrate choline but not by cholinephosphate or CDP-choline. A possible interpretation of channeling is that the three enzymes of this pathway form a complex that is only accessible by choline. According to this hypothesis, all three enzymes of the CDP-choline pathway are expected to localize to the primary site for PC synthesis. The question of why mammalian cells need multiple isoforms of CT specifically localized to various compartments, whereas the presence of a single isoform anywhere in the cell is sufficient for its role for PC synthesis, remains intriguing and unanswered.

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REFERENCES

1. Vance, D. E. (1990) Biochem. Cell Biol. 68, 1151–1165
2. Esken, J. D., and Raetz, C. R. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 5192–5196
3. Cui, Z., Houweling, M., Chen, M. H., Record, M., Chap, H., Vance, D. E., and Kent, F. (1996) J. Biol. Chem. 271, 14668–14671
4. Weinhold, P. A., Rounsifer, M. E., and Feldman, D. A. (1986) J. Biol. Chem. 261, 5104–5110
5. Kalmar, G. B., Kay, R. J., Lachance, A., Aebersold, R., and Cornell, R. B. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6029–6033
6. Wang, Y., Sweitzer, T. D., Weinhold, P. A., and Kent, C. (1993) J. Biol. Chem. 268, 5899–5904
7. Pechel, S. L., Pritchard, P. H., Brindley, D. N., and Vance, D. E. (1983) J. Biol. Chem. 258, 6782–6788
8. Cornell, R., and Vance, D. E. (1987) Biochim. Biophys. Acta 919, 26–36
9. Terce, F., Record, M., Tronchere, H., Ribbes, G., and Chap, H. (1992) Biochem. J. 282, 333–338
10. Houweling, M., Cui, Z., Anfuso, C. D., Bussiere, M., Chen, M. H., and Vance, D. E. (1996) Eur. J. Cell Biol. 69, 55–63
11. Lykidis, A., Murti, K. G., and Jackowski, S. (1998) J. Biol. Chem. 273, 14022–14029
12. Wang, Y., Macdonald, J. L., and Kent, C. (1995) J. Biol. Chem. 270, 354–360
13. Wang, Y., and Kent, C. (1995) J. Biol. Chem. 270, 18948–18952
14. Yang, J., Wang, J., Tseu, I., Kuliszewski, M., Lee, W., and Post, M. (1997) Biochem. J. 325, 29–38
15. Northwood, I. C., Tong, A. H., Crawford, B., Drobnies, A. E., and Cornell, R. B. (1999) J. Biol. Chem. 274, 26240–26248
16. Sweitzer, T. D., and Kent, C. (1994) Arch. Biochem. Biophys. 311, 107–116
17. Davis, R. A., Engelhorn, S. C., Pangburn, S. H., Weinstein, D. B., and Steinberg, D. (1979) J. Biol. Chem. 254, 2010–2016
18. Chen, C., and Okayama, H. (1987) Mol. Cell. Biol. 7, 2745–2752
19. Kent, C. (1997) Biochim. Biophys. Acta 1348, 79–90
20. Bligh, E. G., and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–991
21. Loenning, U. K. (1970) Nature 277, 680–685
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350–4354
23. Jackowski, S. (1996) J. Biol. Chem. 271, 20219–20222
24. Wawra, E., and Wintersberger, E. (1983) Mol. Cell. Biol. 3, 297–304
25. Hoyt, M. A., Totis, L., and Roberts, B. T. (1991) Cell 66, 507–517
26. Goslin, K., Birgbauer, E., Banker, G., and Solomon, F. (1989) J. Cell Biol. 109, 1621–1631
27. Warren, G. (1993) Annu. Rev. Biochem. 62, 323–348
28. Lykidis, A., Baburina, I., and Jackowski, S. (1999) J. Biol. Chem. 274, 26992–27001
29. Wang, Y., Macdonald, J. L., and Kent, C. (1993) J. Biol. Chem. 268, 5512–5518
30. George, T. P., Morash, S. C., Cook, H. W., Byers, D. M., Palmer, F. B., and Spence, M. W. (1989) Biochim. Biophys. Acta 1004, 283–291