Highly Saturated Endonuclear Phosphatidylcholine Is Synthesized in Situ and Colocated with CDP-choline Pathway Enzymes

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Chromatin-associated phospholipids are well recognized. A report that catalytically active endonuclear CTP:choline-phosphate cytidylyltransferase α is necessary for cell survival questions whether endonuclear, CDP-choline pathway phosphatidylcholine synthesis may occur in situ. We report that chromatin from human IMR-32 neuroblastoma cells possesses such a biosynthetic pathway. First, membrane-free nuclei retain all three CDP-choline pathway enzymes in proportions comparable with the content of chromatin-associated phosphatidylcholine. Second, following supplementation of cells with deuterated choline and using electrospray ionization mass spectrometry, both the time course and molecular species labeling pattern of newly synthesized endonuclear and whole cell phosphatidylcholine revealed the operation of spatially separate, compositionally distinct biosynthetic routes. Specifically, endogenous and newly synthesized endonuclear phosphatidylcholine species are both characterized by a high degree of diacyl/alkylacyl chain saturation. This unusual species content and synthetic pattern (evident within 10 min of supplementation) are maintained through cell growth arrest by serum depletion and when proliferation is restored, suggesting that endonuclear disaturated phosphatidylcholine enrichment is essential and closely regulated. We propose that endonuclear phosphatidylcholine synthesis may regulate periodic nuclear accumulations of phosphatidylcholine-derived lipid second messengers. Furthermore, our estimates of saturated phosphatidylcholine nuclear volume occupancy of around 10% may imply a significant additional role in regulating chromatin structure.

Considerable evidence supports the existence of an endonuclear pool of phospholipid, in association with the nuclear matrix and distinct from the nuclear envelope (1–4). This endonuclear phospholipid is remarkable for several reasons. Although all the major membrane phospholipids, predominantly phosphatidylcholine (PtdCho),1 phosphatidylethanolamine (Pt-

dEtn), phosphatidylserine, and phosphatidylinositol (PtdIns), may be present typically at 4–10% of total cell content (2), transmission electron microscopy has failed to reveal endonuclear membranous systems (5). Hence the molecular organization of endonuclear phospholipids in eukaryotic cells is still unclear. Extensive histochemical and cytochemical studies suggest that their spatial distributions overlap that of decondensed chromatin domains (3, 4). A number of in vitro studies suggest a functional relationship between various endonuclear phospholipids and gene expression/transcription (6–10). Moreover, cell studies have shown that the amounts of endonuclear phospholipids change during progression through the cell cycle (4).

The potential physiological importance of intranuclear phospholipid has recently been highlighted by the recognition that the α isoform (CCTα) of CTP:choline-phosphate cytidylyltransferase, the principal regulatory enzyme of PtdCho biosynthesis (11), is confined to the nucleus throughout the cell cycle (12). Furthermore, modified Chinese hamster ovary MT 58 cells (13) expressing a temperature-sensitive mutation of CCTα are not viable when grown at the restrictive temperature (12), suggesting that endonuclear synthesis of PtdCho is essential for cell survival. This hypothesis is supported by the observation that extranuclear PtdCho biosynthesis, which occurs at the endoplasmic reticulum (14) and involves one or both of the β isoform(s) of cytidylyltransferase (CCTβ-1, CCTβ-2) (15), cannot rescue mutant cells grown at the restrictive temperature (12). However, the mechanism whereby endonuclear PtdCho biosynthesis regulates cell proliferation is not clear, and as yet there has been no direct demonstration of the intact pathway within the nucleus. One possible role for endonuclear PtdCho biosynthesis might be to regulate the periodic accumulations of diacylglycerol (DAG) within the nucleus that are functionally linked to cell proliferation (16). Mobilization of DAG in the nucleus is bipartite (17) and involves two pools: one derived from predominantly unsaturated PtdIns and one derived from largely saturated PtdCho. Accordingly, a nuclear CDP-choline pathway for endonuclear PtdCho biosynthesis could regulate the nuclear DAG content by recycling endonuclear PtdCho-derived DAG together with choline and or phosphocholine.

Consequently, we sought to determine whether expression of CCT activity within the nucleus does indeed represent part of an intact compartmentalized CDP-choline pathway for intranuclear PtdCho biosynthesis. First, we measured activities of individual CDP-choline pathway enzymes to establish the potential capacity for PtdCho synthesis within the nucleus. Subsequently, we employed electrospray ionization mass spec-

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1 The abbreviations used are: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdIns, phosphatidylinositol; CCT, CTP:choline-phosphate cytidylyltransferase (EC 2.7.7.15); DAG, diacylglyc-

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trometry (ESI-MS) of PtdCho isolated from cells cultured with deuterated choline to quantify the rate of endogenous PtdCho synthesis. ESI-MS also permitted a detailed analysis of the molecular species compositions of endogenous and newly synthesized molecular species of PtdCho within the nucleus.

**MATERIALS AND METHODS**

Stock IMR-32 human neuroblastoma cells, obtained from Imperial Laboratories (Andover, Hampshire, UK), were maintained in monolayer culture in RPMI 1640 containing 10% fetal bovine serum and penicillin/streptomycin/amphotericin (Life Technologies, Inc.) at 37 °C in 5% (v/v) CO₂. Sterile Hank's balanced salt solution (HBSS) and HBSS without Ca²⁺/Mg²⁺ was also obtained from Life Technologies, Inc. Electrophoresis chemicals were from Promega (Southampton, Hampshire, UK). PtdCho standards were obtained from Sigma. Specialist chemicals for electron microscopy were from Agar Scientific (Stansted, Essex, UK), and other chemicals were of AnalaR grade from Merck or from Sigma-Aldrich unless otherwise stated. Phosph[14C]methylcholine and CDP-[14C]methylcholine were supplied from Merck or from Sigma-Aldrich unless otherwise stated. (Stansted, Essex, UK), and other chemicals were of AnalaR grade from Merck or from Sigma-Aldrich unless otherwise stated.

**Preparation of Membrane-depleted Nuclei—**Trypsinized, subconfluent IMR-32 cells (2–6 × 10⁶ cells/experiment) were washed twice with 10 μl of HBSS without Ca²⁺/Mg²⁺ and diglycidyl ether:nonenyl succinic anhydride:dimethylamino ethanol (7:2:1 with 0.5% w/v NaI). Following fragmentation with argon gas, the 193 parent scans the corresponding peaks were 9 m/z values. The PtdCho species were expressed as a percentage of the total present in the sample. The formula for reduced response with increased m/z values was determined during nuclei preparation. Data were acquired and processed using MassLynx NT software. After conversion to centroid format according to area and correction for ¹³C isotope effects and for reduced response with increasing m/z values, the PtdCho species were expressed as a percentage of the total present in the sample. The formula for reduced response with increased m/z values was determined experimentally for PtdCho as follows: a = 2 × 10⁻³ b × 3.9973, where a reduced response factor relative to a value of 1.00 for PtdCho 14:0/14:0, and b = m/z value. In the m/z = 184 parent scans, desaturated species occurred at the following m/z values: 16:0 alkyl/14:0, m/z = 683; 16:0/14:0, m/z = 707; 16:0 alkyl/16:0, m/z = 721; 16:0/16:0, m/z = 755; 16:0 alkyl/16:0, m/z = 748; 16:0 alkyl/16:0, m/z = 769; 16:0 alkyl/16:0, m/z = 781; 16:0 alkyl/16:0, m/z = 803. Peak identifications were confirmed by product ion scanning of fatty acyl groups by tandem MS/MS. ESI-MS and ESI-MS/MS of PtdEtn from whole cell or nuclei was performed in methanol/chloroform:water (7:2:1 with 0.5% w/v Na₂). Following fragmentation with argon gas, sodium adducts of PtdEtn lost a neutral fragment of m/z = 141, corresponding to the protonated phosphoethanolamine headgroup. Sequential neutral losses of m/z = 184 permitted determination of PtdEtn species present within the samples. CDPE-Choline Pathway Enzyme Determinations—CD activity was determined by the method of Ishidate and Nakazawa (25). C T assay was performed as previously described (26), with some modifications. Charcoal washes with water were increased to five, and elution washes in ethanol/ammonia were increased to three; all were performed at room temperature. Lipid-stimulated activity was measured in the presence of pholipid vesicles prepared as described by Wei and Feldman (27). ADP (6 mM) and additional MgCl₂ (3 mM) were included to inhibit intrinsic nucleotidase activity (27), and all determined activities were corrected using CDPE-methyl-¹⁴C]-choline recovery standards in each batch of assays (27). CDPE-choline:1,2-diaclylglycerol choline phosphotransferase (EC 2.7.8.2) activities were determined as reported previously (28) but included additional lipid blanks for nuclear activity determinations to correct for high endogenous DAG. All activities were expressed in relation to a unit number of cells or isolated nuclei (1 × 10⁷) as determined during nuclear preparation.
Nuclear Matrix Phosphatidylcholine Synthesis

The activities of choline kinase (CK), CCT, and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) were determined in post-mitochondrial (10,000 × g; 10 min) supernatants of cells and of a proportion of nuclei stripped of their nuclear membranes from the same flask as described under "Materials and Methods." Each value represents the mean activity corrected for cell/nuclei count (n = 5) ± S.D.

TABLE I

| Enzyme                  | Extranuclear Activity | Nuclear Activity |
|-------------------------|-----------------------|------------------|
|                         | nmol synthesized/min/10^6 nuclei |                 |
| CK                      | 1.57 ± 0.83           | 0.18 ± 0.14      |
| CCT without PtdCho/oleate| 0.98 ± 0.70           | 0.33 ± 0.09      |
| CCT with PtdCho/oleate  | 15.14 ± 4.02          | 0.47 ± 0.18      |
| CPT                     | 2.72 ± 0.95           | 0.40 ± 0.08      |

Exclusion of both endoplasmic reticulum and nuclear envelope membranes from experimental nuclear matrix preparations is an essential requirement for quantification of chromatin-associated phospholipid compositions and biosynthetic capacities. Detergent-based extraction protocols have been successfully exploited by other groups studying aspects of nuclear phosphoinositide metabolism and can be designed either to retain (29) or remove (2, 16, 30) the nuclear membrane from isolated nuclei. Triton X-100 was selected for the present study based upon a report that concentrations above 0.04% (w/v) completely removed the nuclear envelope from isolated nuclei of rat liver (2). Low and high power electron microscopy clearly showed a typical limiting double bilayer membrane in whole IMR-32 cell nuclei (Fig. 1, a and b) that was absent from nuclei isolated in the presence of 0.5% Triton X-100 (Fig. 1, c and d). Strong evidence for removal of endoplasmic reticulum and weaker evidence of nuclear envelope membrane removal were also provided by the lack of reactivity to anti-β-tubulin upon Western blotting of isolated nuclei (Fig. 1e). Absence of β-tubulin has been widely accepted as indicative of pure nuclear matrix preparations in previous studies (16, 19, 20), although nuclear envelope content below the level of immunoreactivity cannot be excluded by such methodology. This procedure resulted in a reproducibly high recovery of membrane-free nuclei (67.3 ± 6.7% (mean ± S.D.; n = 18). It was highly dependent upon exclusion of Ca^{2+}/Mg^{2+} from the extraction medium, because the presence of these ions induced a denaturing aggregation of nuclear matrix preparations.

The capacity of the nuclear matrix of IMR-32 cells to sustain a compartmentalized synthesis of PtdCho was determined by evaluating the presence, and assaying the activities, of each of the three enzymes of the CDP-choline pathway, namely choline kinase (EC 2.7.1.32), CCT, and CDP-choline:1,2-diacylglycerol cholinephosphotransferase (Table I). Endonuclear choline kinase activity was 10.3% that of the whole cell, whereas endonuclear CCT without PtdCho/oleate stimulation was 25.5% of whole cell activity. This endonuclear CCT activity was not stimulated by incubation with PtdCho/oleate, in contrast to the 15-fold stimulation of the extranuclear enzyme by lipid. The absence of an effect of exogenous lipid on nuclear CCT may have been due either to an inability of the PtdCho/oleate vesicles to penetrate the nuclear matrices or to residual Triton X-100, which may compromise lipid stimulation. Chromatin-associated CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity comprised 12.8% of whole cell activity. Likewise, it is probable that Triton X-100 residue and inability of exogenous DAG vesicles to penetrate the nuclear matrices may have diminished CDP-choline:1,2-diacylglycerol cholinephosphotransferase activity determinations in this compartment. Notwithstanding the possibility that choline kinase activity may also have been affected by Triton X-100 exposure, substrate accessibility was less likely to be problematic.

Whereas the enzyme activities we observed demonstrate the capacity for in vitro endonuclear transformation of each of the PtdCho intermediates to their proximal biosynthetic products, they do not prove the existence of functionally compartmentalized pathways in intact cells. Consequently we used deuterium-enriched choline-d_9 as a labeled substrate in conjunction with ESI-MS to probe PtdCho biosynthesis (23). The ratio of endogenous to newly synthesized PtdCho molecular species was determined by incubating IMR-32 cells with choline-d_9 and then extracting the PtdCho from isolated nuclei and from whole cells. Sequential scans of the parents of fragments with m/z of 184 and 193 were used to quantify endogenous and newly synthesized PtdCho species (23) (Fig. 2, a–d). Incorporation of choline-d_9 into PtdCho from whole cells was readily detectable by 10 min and linear for up to 3 h (Fig. 3b), after which time 8.6% of whole cell PtdCho contained the d_9 headgroup (Fig. 3a). The time scale for the incorporation of choline-d_9 into the endonuclear PtdCho was equally rapid; the parents of the m/z 193 species were detectable within 10 min (Fig. 3b). However, a constant rate of labeled choline incorporation was only achieved after the first hour of supplementation (Fig. 3b), presumably representing the time required for equilibration of the d_9 substrate into the endonuclear choline pool.

ESI-MS analysis permitted the analysis of the molecular composition of both endogenous and newly synthesized PtdCho (Fig. 4, a and b). By using PtdCho 14:9/14:0 as an internal standard, we were able to determine an endonuclear PtdCho concentration of 3.55 ± 1.48 nmol/10^6 nuclei (corresponding to 3.55 ± 1.48 × 10^{-15} mol/nucleus). This represented 5.95 ± 0.69% (n = 8; mean ± S.D.) of the PtdCho present in the entire cell. Analysis of the species composition of the endonuclear and whole cell PtdCho obtained from the parents of the m/z 184 fragment (Fig. 4a) revealed that endonuclear PtdCho was enriched in species where both fatty acids were saturated.

**FIG. 1. Purity of nuclei preparations.** Electron micrographs of whole IMR-32 human neuroblastoma cells (a, scale bar 10 μm) show at higher magnification (c, scale bar 0.5 μm) the classical double-layer nuclear envelope. Purified, washed nuclei pellets (b, scale bar 10 μm) showed no nuclear envelope at the corresponding higher magnification (d, scale bar 0.5 μm). Western blots of nuclei (N) and whole cell (C) fractions probed with monoclonal anti-β-tubulin (e) at equivalent cell loading displayed no immunoreactivity in the nuclear fraction, consistent with removal of endoplasmic reticulum and minimal nuclear envelope contamination (21, 22).
The proportion of such disaturated PtdCho to total PtdCho was 16.8% for whole cells (Fig. 4a) but in contrast was 60.3% for endonuclear PtdCho (Fig. 4b). PtdCho species with polyunsaturated fatty acid chains, particularly 20:4 or 22:6, comprised 27.9% of the total PtdCho from whole cells (Fig. 4a) but were below the limits of detection in the endonuclear PtdCho pool (<0.5% of the largest peak). This observation provided an unexpected additional proof of the purity of the nuclear matrix preparations, because PtdCho species containing 20:4 fatty acids are a major component of the nuclear envelope (31, 32). Examination of the parents of the m/z 193 fragment (Fig. 2, a and b) suggested that after 3 h both nuclear and whole cell pathways synthesized PtdCho molecular species with a similar composition to those of their respective endogenous PtdCho pools. However, closer examination of the data revealed subtle differences (Fig. 4, a and b). Whereas whole cell PtdCho synthesis was indeed similar to the endogenous PtdCho after 3 h, consistent with completion of any remodeling within that time scale, comparison of newly synthesized endonuclear PtdCho and the corresponding endogenous pool suggested that endonuclear remodeling mechanisms probably continue after 3 h. Specifically, 53.6% of newly synthesized endonuclear PtdCho after 3 h was disaturated (Fig. 4b) compared with 60.3% of endogenous composition (Fig. 4a), and the distribution between saturated species differed. Both 16:0/16:0 and 16:0/18:1 species were more abundant in newly synthesized endonuclear PtdCho. Moreover, the pattern of endonuclear PtdCho synthesis seen after 10 min of choline-d<sub>9</sub> supplementation (Fig. 5a) implies progressive remodeling of newly synthesized PtdCho to more saturated species. In agreement with this suggestion, the acyl-CoA pool associated with chromatin is known to be more saturated than the corresponding acyl-CoA of the whole cell (33). Additionally, acyl-CoA synthetase activity associated with the nuclear matrix has the lowest apparent K<sub>m</sub> for 16:0, indicating a preference for acylation of this acid inside the nucleus (34). Consequently, the substrates necessary for CoA-dependant remodeling of endonuclear PtdCho to more saturated species are probably present within the nuclear matrix. PtdCho species containing 20:4 or 22:6 comprised 33.6% of whole cell PtdCho synthesis after 3 h but were essentially absent from nuclear PtdCho synthesis (Fig. 4b) even at 10 min (Fig. 5a). This suggests that if they are synthesized within the nucleus de novo, then they are rapidly remodeled to more saturated species.

The enrichment of disaturates in newly synthesized endonuclear PtdCho after 10 min (Fig. 5a) compared with whole cell PtdCho synthesis (Fig. 4b) and the rapidity of synthesis are themselves very good evidence for the existence of a compartmentalized PtdCho synthesis pathway within the nucleus. It is possible that PtdCho synthesized on the endoplasmic reticulum could be the source of newly synthesized endonuclear PtdCho after 10 min of incubation with choline-d<sub>9</sub> (Fig. 5a). However, this would require very rapid selective transport of saturated PtdCho species from the endoplasmic reticulum, possibly involving an as yet unrecognized saturated PtdCho transfer protein, across the nuclear envelope and into the nuclear matrix. No such mechanisms have been demonstrated for PtdCho transport that are both sufficiently selective and rapid, and consequently endonuclear PtdCho synthesis is the simplest explanation for our results. The data presented here, together with CDP-choline pathway enzyme activities and the recognized requirement for enzymatically active (endonuclear) CCTα (12), argue strongly for the existence of a functional pathway for the synthesis of endonuclear PtdCho in the intact cell.

Our ESI-MS studies also enabled the analyses of all other endonuclear phospholipids. Simultaneous use of PtdEtn and PtdOH internal standards permitted the determination of the concentrations as well as the species of endonuclear PtdEtn and PtdOH pools. Endonuclear concentrations of PtdEtn and PtdOH were 0.54 ± 0.34 and 0.15 ± 0.003 nmol/10<sup>8</sup> nuclei, respectively. In contrast to the dramatic difference between PtdCho compositions between the whole cell and nucleus, the compositions of PtdOH and PtdEtn were essentially identical between whole cell and endonuclear pools. PtdEtn isolated from both sources was composed largely of monounsaturated species (results not shown), whereas PtdEtn was highly unsaturated (Fig. 6). For both whole cell (Fig. 6a) and endonuclear (Fig. 6b) pools, PtdEtn species containing 20:4 or 22:6 polyunsaturated chains contributed >80% of the total, with relatively minor variation seen between the fractional contents of individual unsaturated species. Whereas the highly saturated nature of endonuclear PtdCho might be expected to exist in a rigid gel phase, the presence of the unsaturated PtdEtn is likely to render endonuclear lipid a more fluid and metabolically active phase at physiological temperatures. The presence of PtdEtn in the endonuclear phospholipid pool, with a high degree of chain unsaturation, is also probably necessary for CCT activity. A recent study showed that CCTα activity in vitro was modulated by the amount of elastic energy stored in a bilayer membrane (35), which in turn was determined by the lipid composition of that membrane. Whereas CCTα was essentially inactive in membranes of saturated PtdCho, introduction either of PtdCho with unsaturated chains or of PtdEtn species led to an increased activity. These lipids are known as Type II lipids because they have negative spontaneous curvatures. From our ESI-MS data we estimate that some 23 mol % of the endo-

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**Fig. 2.** ESI-MS of PtdCho from whole IMR-32 cells and nuclear matrices after 3 h of incubation with choline-d<sub>9</sub>. a, scan of parents of fragment with m/z = 184 (choline headgroup); endogenous whole cell PtdCho. Selected disaturated species are indicated by arrows. b, scan of parents of fragment with m/z = 193 (choline-d<sub>9</sub> headgroup); newly synthesized whole cell PtdCho. c, scan of parents of fragment with m/z = 184; endogenous nuclear matrix PtdCho. Disaturated molecular species are indicated by arrows. d, scan of parents of fragment with m/z = 193; newly synthesized nuclear matrix PtdCho.
nuclear phospholipids were Type II lipids, a value consistent with support for CCTa activity.

Concentrations of endonuclear PtdCho, PtdEtn, and PtdOH determined from the ESI-MS studies were used to estimate the volume that these lipids would occupy in the nucleus (36). The molecular volume \( v \) of a phospholipid can be estimated from

\[
v = 2v_{CH} + (n_{CH} + n_{CH}^2)v_{CH} + n_{CH}^2v_{CH} + v_{polar} \quad \text{(Eq. 1)}
\]

where \( v_j \) represents the volume of a CH\(_3\), CH\(_2\), or CH unit or of the polar headgroup, and \( n_i \) represents the number of CH\(_x\) or CH units in each chain \( (i = 1, 2) \). The following values, appropriate for phospholipids in an L\(_2\) phase, were used in the calculation: \( v_{CH} = 51.0 \ A^3; v_{CH_2} = 25.5 \ A^3; v_{CH} = 20.5 \ A^3; v_{polar}(PtdCho) = 260 \ A^3; v_{polar}(PtdEtn) = 246 \ A^3; v_{polar}(PtdOH) = 135 \ A^3 \). The amounts of each endonuclear species as determined by ESI-MS were used to estimate the volume occupied by each species \( (V_{PtdCho} = 2.74 \times 10^{-18} \ m^3; V_{PtdEtn} = 4.40 \times 10^{-19} \ m^3; V_{PtdOH} = 9.95 \times 10^{-21} \ m^3) \), which gives a
total volume of $3.15 \times 10^{-18}$ m$^3$. This volume is the minimal volume that would be occupied by the lipids if they were packed into a single bilayer. To allow for hydration the total volume should be increased by a factor of 1.5 to 2. The volume of a typical IMR-32 nucleus was estimated to be $3.9 \times 10^{-17}$ m$^3$ by assuming an oblate shape, consistent with transmission electron microscopy observations, with semi-axes 1.5, 1.25, and 5 $\mu$m in length.

Assuming that these lipids are fully hydrated, such calculation leads to a volume between $4.7 \times 10^{-18}$ and $6.3 \times 10^{-18}$ m$^3$, which corresponds to 12–16% of the nuclear volume for IMR-32 nuclei ($3.9 \times 10^{-17}$ m$^3$). For comparison, the $6 \times 10^9$ nucleotide pairs in human DNA would occupy a minimum theoretical volume of $6.86 \times 10^{-18}$ m$^3$. Because the total mass of histones in chromatin is roughly equal to the mass of DNA, the volume occupied by the 48 chromosomes is estimated to be of the order of $1.5 \times 10^{-17}$ m$^3$ ($\sim 38.5\%$ of the nuclear volume). The maximum amount of water available to hydrate endonuclear lipids, estimated by subtracting the combined chromosome and lipid volumes from the nuclear volume (and assuming no other organic species or ions are present), is between $1.7 \times 10^{-11}$ and $1.9 \times 10^{-11}$ g. By using a conservative average molecular weight of 700 for the lipid, we estimate that at the very least the endonuclear phospholipids we studied were present at an effective concentration of about 10% (weight of phospholipid/weight of free water). The relatively high phospholipid to water ratios inside the nucleus suggest that these lipids are likely to be present as large complex aggregates and possibly even as liquid crystalline phases.

Because the synthesis of extranuclear PtdCho varies with the cell cycle (37), we sought to establish whether the synthesis of endonuclear PtdCho and species composition were constitutive or subject to temporal regulation. Proliferating IMR-32 cells were induced to enter a pseudo-differentiated state, $G_0$, by culturing them in serum-depleted medium (0.5% FCS) for 72 h. This halted proliferation and extension of neurite-like structures was observed. Quiescent cells were susceptible to mitogenic reentry into the cell cycle by supplementation with 10% FCS (as determined by growth cone collapse, cell rounding, and proliferation assays). Following the growth arrest mediated by serum depletion, the basal whole cell PtdCho synthesis, assayed during 3 h of incubation with choline-$d_9$, fell to 28% of control values (Fig. 3a). Upon supplementation with 10% FCS, the whole cell PtdCho synthesis rose to 47% of control (Fig. 3a), presumably reflecting increased PtdCho synthesis during re-entry into the cell cycle. In the case of the endonuclear PtdCho, the basal synthesis following growth arrest was 20% of control, whereas reentry into the cell cycle led to an increase in synthesis to 40% of control. Minor changes in whole cell PtdCho and endonuclear PtdCho species were observed following serum depletion, and these were reflected by the altered species composition of the newly synthesized PtdCho. However, the proportion of disaturated species in both endogenous and newly synthesized endonuclear PtdCho pools remained remarkably constant, indicating that this unusual acylation/alkylation pattern was under tight homeostatic control. Cornell and co-workers (38) reported that mitogenic stimulation of serum-starved IIC9 cells by serum supplementation was accompanied by a
translocation of nuclear CCTα to the endoplasmic reticulum, although demonstration of nuclear confinement of CCTα throughout the cell cycle (12) apparently contradicts that result. Explanations of the discrepancy have been attributed either to insufficient specificity of CCT antibodies or cell type-specific change (12). Our data did not include measures of CDP-choline pathway enzyme activities during extracellular serum manipulation and cannot inform that debate except insofar as we show that both nuclear and whole cell PtdCho synthesis increased in serum-starved IMR-32 cells following serum resupplementation. It would seem unlikely, therefore, that the nuclear matrices would act solely as a reservoir for exportable CCT destined for the endoplasmic reticulum of IMR-32 cells at a time when endonuclear PtdCho synthetic flux doubles.

The fundamental function for endonuclear PtdCho synthesis is not clear. It is very unlikely that this quantitatively minor pathway for PtdCho synthesis contributes significantly to bulk membrane phospholipid generation within the cell. Equally, the evidence suggests that it is unrelated to the synthesis of new nuclear envelope generated as part of cell division, not least because of the highly unsaturated nature of these membranes (31, 32). Three possibilities, however, are advanced. First, CCT, as the principal regulatory enzyme of PtdCho biosynthesis (1), produces CDP-choline for the proximal enzyme of the pathway, CDP-choline:1,2-diacylglycerol cholinephosphotransferase, thereby controlling pathway flux. The other substrate of CDP-choline:1,2-diacylglycerol cholinephosphotransferase is DAG. Accordingly, our data would support a role for an endonuclear CDP-choline pathway in regulating endonuclear disaturated DAG content. Temporally distinct periodic nuclear accumulations of unsaturated DAG from PtdIns (16, 39) and saturated DAG from PtdCho (17) are recognized. CCTα activity is clearly essential for cell survival (12), whereas overexpression of a nuclear-targeted DAG kinase ablates nuclear DAG accumulations by converting them to PtdOH and halts cell growth (16). Control of flux through endonuclear PtdCho synthesis at key points in the cell cycle may therefore serve to regulate this potential endonuclear lipid second messenger, possibly by preventing its conversion into saturated, biologically active PtdOH (40). Second, the highly saturated nature of endonuclear PtdCho, together with the estimations of volume occupancy that we have made, may reflect a further specialized structural role for endogenous PtdCho within the nuclear matrix. Finally, the same saturation offers the possibility of a third role for saturated endogenous PtdCho, the prevention of the propagation of oxidative damage within nuclei. Free radical generation in close proximity to the genome is clearly undesirable. Moreover, the presence of significant amounts of polyunsaturated lipids in the nuclear envelope and as part of the endonuclear PtdEtn (Fig. 6b), PtdOH, and PtdIns (39) dispersed within the nuclear matrix and in close association with DNA poses significant risk of amplification of reactive oxygen species. Saturated PtdCho in the nuclei may provide a means of halting oxidative damage before it overwhelms endogenous antioxidant capacity.
The presence of endonuclear CCTa appears to be universal in a range of cultured cell types (12), and similarly, highly saturated endonuclear PtdCho synthesis may not be confined to IMR-32 neuroblastoma cells. Preliminary data from our laboratories\(^2\) show that human HaCaT keratinocytes and U937 cells likewise synthesize highly saturated endonuclear PtdCho. Both the structural possibilities and the intimate association of saturated endonuclear PtdCho synthesis with the process of cell proliferation render this compartment of phospholipid metabolism a challenging area of future research.

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\(^2\) A. N. Hunt, G. T. Clark, and A. D. Postle, unpublished observations.