Evidence for a Unique Profile of Phosphatidylcholine Synthesis in Late Mitotic Cells

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ABSTRACT Evidence is presented that the structural rearrangements in late mitosis are accompanied by an alteration in membrane lipid synthesis. This evidence was derived from analyzing phospholipid classes after rapid-labeling, as well as from determining the intracellular site of incorporation of choline by HeLa S3 cells as they progressed from metaphase into early interphase (G1). Compared with postmitotic cell data, the recent mitotic cell data indicate a specific two- to threefold increase in the net synthesis of phosphatidylcholine (PC) species, which appeared to contain the more saturated fatty acids. Since this was observed with glycerol, choline, and orthophosphate labelings, and not with methyl labeling, it appears that the CDP-choline plus diacylglycerol pathway rather than the phosphatidylethanolamine to PC pathway was augmented. Electron microscope autoradiography of anaphase, telophase, and early G1 cells demonstrated that the reformed nuclear envelope was the incorporation site of a significant proportion of the newly synthesized PC. This incorporation occurred by early telophase prior to chromosome decondensation. The potential significance of PC metabolism with regard to membrane rearrangements, such as nuclear envelope reformation, is discussed.

The late mitotic cell undergoes a series of dynamic ultrastructural changes (11, 19) resulting in progeny cells in which macromolecular synthesis (16, 22, 23) has been re-established. Among the ultrastructural events are several that involve the membranous components of the cell, such as reforming the nuclear envelope (NE), rearranging the endoplasmic reticulum (ER), and breaking and resealing the cytoplasmic membrane. Although ultrastructural changes in mitosis have long been recognized, the biochemical basis of most of these events, except for those involving the spindle apparatus (for a review see reference 18), is largely unknown. For example, this is the case for NE reformation, which is an early event in telophase. It is thought that membrane fragments for this reformation are, at least in part, carried through cell division at the telomeres of chromosomes (10). Recently, it has been argued that peripheral dense lamina polypeptides (putatively, NE components) play a role in this process (14). These polypeptides, which have been designated lamins A, B, and C, apparently become less phosphorylated during nuclear reformation.

What role, if any, newly synthesized membrane lipids play in either nuclear or cytoplasmic membranous events cannot be evaluated at the present time because there is no information that can be associated unequivocally with cells in late stages of mitosis. The available data, on the other hand, have been obtained from partially synchronized cells and indicate that the incorporation of membrane lipids into the NE occurs only after the cell has entered interphase (G1) (8) and, thus, suggest that newly synthesized lipids are not involved in this process.

In this paper, however, data are presented that suggest a specific role for a particular class of membrane lipids in late mitotic events. We have determined the amount and the profile of phospholipid synthesized in tightly synchronized populations, and the intracellular site of incorporation of one class of these membrane components. Specifically, aspects of phospholipid biosynthesis were explored in the human derived cell line, HeLa S3, by following the incorporation of radioactive orthophosphate, glycerol, choline, and methionine under conditions of "rapid-labeling." For this analysis, metaphase cells were collected and then, by incubation at 37°C, were permitted to traverse through the final stages of mitosis. In addition, the individual stages of late mitosis were analyzed for the intracellular distribution of choline incorporation by electron microscope autoradiography (EMAR). Our data indicate that late mitotic cells have an augmented phosphatidylcholine (PC) biosynthetic activity, in particular, and that a substantial portion of this newly synthesized PC can be visualized in association with the NE by early telophase before chromosome decondensation.

MATERIALS AND METHODS

Cell Culture and Synchronization: HeLa S3 cells were maintained at 37°C in suspension culture in Eagle's minimal essential medium (Gibco Laboratories, Grand Island, NY) supplemented with 7% calf serum and 2 mM glutamine.
Populations of cells in metaphase were obtained by selective detachment after a double-thymidine blockade as previously described (16). These preparations had a metaphase index of at least 88% and a mitotic index of 94%.

Populations of cells that progressed through metaphase, anaphase, and telophase, and into early G1, and populations of cells in mid and late G1 periods of the cell cycle were obtained by incubation of the collected metaphase cells at 37°C for 90 min (early G1), 4 h (mid G1), or 6.5 h (late G1), respectively. Late mitotic cells were obtained with shorter incubation periods as indicated in the appropriate figure legends. We have demonstrated previously that cells synchronized in metaphase begin to enter S phase 7-8 h after selective detachment (22).

Labeling of Cellular Phospholipid Components: Populations of synchronized cultures were exposed to radioactive choline, glyceral, orthophosphate, or methionine under conditions that maximized linear uptake. Labeling in each instance was done for 90 min, which was equal to that time needed for populations of metaphase cells to enter early G1. These conditions for "rapid-labeling" permitted sufficient incorporation of label for subsequent analysis of the extractable classes of phospholipids.

Cells were exposed to radioactivity at a concentration of 1 x 10^6 cells/ml in prewarmed medium containing 7% dialyzed calf serum and 2 mM glutamine at 37°C. To label with radioactive phosphate, the medium was phosphate free and contained 0.15 mM carrier-free [32P]orthophosphate (New England Nuclear Boston, MA). To label with glyceral or choline, the medium contained one-tenth the normal concentration of choline and 10 mM choline chloride (4.2 Ci/mmol, New England Nuclear). To label with methionine, the medium was methionine free and contained 25 mM carrier-free [3-3H]methionine, (15 Ci/mmol, New England Nuclear). Some experiments were performed with delipidized serum.

Radioactivity: Species of PC (lecithin) with different degrees of unsaturation were analyzed by argentation chromatography essentially as described by Arvidson (3). Samples of PC were first eluted with chloroform/methanol (2:1 vol/vol) from the aforementioned silica gel and were then fractioned on plates (20 x 20 cm) covered with a 0.6-mm thick layer of silica gel H-silver nitrate at a 4:1 ratio (w/w). Prior to use, plates were activated at 195°C for 4 h and stored over P2O5 in a vacuum desiccator. Eluted samples and 0.25 mg each of α- and β-dipalmityl lecithin (Applied Science Div., Milton Roy Co., Laboratory Group, State College, PA), α-dioleoyl lecithin (Applied Science Div., Milton Roy Co., Laboratory Group), α,ω-dilinoleoyl lecithin (Supelco, Inc., Bellefonte, PA), and α,ω-diarachidonyl lecithin (Supelco, Inc.) were applied to individual 1-cm lanes, and chromatography was performed in a solvent system composed of chloroform/methanol/water (60:30:5 vol/vol). Radioactivity was located in successive 5-mm fractions as described above. The marker PC were located by exposing the chromatographic plate to ultraviolet light after spraying with a 0.2% solution of dichlorofluorescein in ethanol.

Determination of Phospholipid Phosphate: Phospholipid phosphate (inorganic phosphate [Pi]) assays were performed spectrophotometrically on dried chloroform/methanol extracts essentially as described by Chen et al. (7). Inorganic phosphate was converted to micrograms of phosphorid by multiplying by a factor of 25.

Electron Microscope Autoradiography: After exposure to radioactivity, preparations of mitotic cells were washed and then fixed in 0.16 M cacodylate buffer containing 0.007 M MgCl2 and 5% glutaraldehyde, stained, dehydrated in an ethanol or an acetone series, and embedded in EPON as previously described (24). After ethanol dehydration, cells retained 40-50% of the radioactivity and, after acetone dehydration, 90-95% of the radioactivity was retained. Either procedure permitted an autoradiographic analysis. Coating sections of ~1,000  with Ilford L4 emulsion (Ilford Ltd., Essex, England) and developing with Microdol X (Eastman Kodak Co., Rochester, NY) were carried out as previously described (24). Sections were then stained with aqueous uranyl acetate and lead citrate, and examined with a Philips (Philips Electronic Instruments, Inc., Mahawah, NJ) 400 transmission electron microscope operating at 80 kV.

RESULTS

Augmented Incorporation of Label in Cells Progressing from Metaphase to Interphase

To investigate aspects of the biosynthesis of new membrane lipids during the metaphase (met) to interphase (G1) transition in cultured HeLa cells, we have followed the labeling of phospholipids with radioactive choline, glyceral, orthophosphate, and the methyl group of methionine. Initial experiments indicated that metaphase cells suspended in phospholipid labeling media passed through anaphase to telophase and into early G1 with the same kinetics as mitotic cells suspended in complete medium at 37°C (Fig. 1 inset). These early G1 cells contained roughly one-half of the metaphase cell content of protein and phospholipid (Fig. 1.4), which by late G1 (6.5 h after synchronization), had increased ~30%.

When cells were exposed to each radioactive label under these conditions, acid-insoluble radioactivity accumulated linearly for at least 90 min (Fig. 1 B). As expected, essentially all the incorporated choline and glyceral were extractable into chloroform/methanol, whereas ~20% of the radioactive orthophosphate and ~30% of the methyl group of methionine were extractable into this lipid solvent. Thus, our initial data indicated that late mitotic cells were active in membrane lipid synthesis.

To determine whether residual protein synthesis during late mitosis, which represents ~10-20% of interphase levels (21), and/or the restitution of protein synthesis to interphase levels in early G1 had any effect on this incorporation, we performed experiments in the presence of an inhibitor of protein synthesis (Fig. 1 B). It was observed that, regardless of the label, incorporation was independent of concomitant protein synthesis since levels of incorporation in the presence of inhibitor were essentially identical to those observed in the absence of inhibitor. Typical data obtained, e.g., with methionine labeling, are presented in Fig. 1 B. Late mitotic stages apparently
were equally active in phospholipid synthesis because when cells were exposed to 10-min pulses of radioactive choline or glycerol the level of incorporation was nearly identical throughout the 90 min-incubation period after synchronization in metaphase (data not shown).

To evaluate more thoroughly this apparent synthesis by late mitotic cells, we quantitated the incorporation with respect to cells that had completed mitosis (Table I). It was observed that incorporation of phosphate, glycerol, and choline label into extractable phospholipid was maximal during the metaphase to interphase period (Table I), and that by late G1 this capacity had decreased ~40~50%. In contrast, no quantitative difference in the incorporation of the methyl group of methionine into extractable cellular phospholipids was noted among any of the synchronized populations.

When the cellular soluble (water-acid) radioactivity for each label was determined, similar amounts of radioactivity were recovered from cells that had progressed from metaphase to interphase, and from mid and late G1 (postmitotic) cells. Thus, although the cytoplasmic membrane of the metaphase cell has unique properties (4, 5), the increased incorporation of these precursors into cellular phospholipids in the met to G1 transition did not appear to represent increased transport of extracellular radioactive precursor.

### Thin-layer Chromatographic Analysis of Phospholipid Synthesis

To determine the classes of phospholipids synthesized in mitotic and postmitotic times, we analyzed the incorporation of each precursor by two-dimensional thin-layer chromatography. The chromatographic system separated phospholipids into 12 classes, e.g., the major choline-containing classes, lysophosphatidylcholine (LPC), PC, and sphingomyelin (SPH) were displayed clearly. When mitotic cells were labeled with either radioactive glycerol or orthophosphate, radioactivity was recovered in each phospholipid class, indicating that the met to G1 transition was active in the total synthesis of phospholipids. As expected, late mitotic cells exposed to radioactive methionine or choline resulted in the recovery of label in LPC, SPH, and PC.

Because of the limited number of cells obtained in the metaphase synchronization procedure, it was necessary to add carrier lipid to each extract to locate individual phospholipid classes. Thus, these data generally were analyzed on the basis of the relative amount of radioactivity recovered. This revealed a disproportionate amount of radioactivity in PC in some experiments (Table II). For example, after labeling with radioactive phosphate (Table II), met to G1 cells, as compared with mid and late G1 cells, yielded ~2.3-fold increase in the relative recovery of radioactivity in PC. Also, with glycerol labeling (Table II) an increase in the relative recovery of radioactivity in PC was noted. Labeling with methionine or choline resulted in a similar distribution of radioactivity regardless of labeling time after synchronization. For example, after choline labeling, ~83, 13, and 1.3% of the label were recovered in PC, LPC, and SPH, respectively; and after methionine labeling was done in the presence of cycloheximide. Inhibitor and radioactivity were added simultaneously in metaphase populations; in mid and late G1 populations, inhibitor was added 15 min prior to the radioactivity. Chloroform/methanol-soluble and TCA-water-soluble fractions were prepared from each population, and the amount of radioactivity or radioactivity was determined as described in Materials and Methods. Values for the chloroform/methanol-soluble fraction are presented (see text) and represent the average of three determinations. For comparison, the data obtained with the metaphase population (met to G1) have been given a value of 100%.

### Table I

**Quantitation of Incorporation of Lipid Precursor**

| Cell stage     | Orthophosphate | Glycerol | Choline | Methionine |
|----------------|----------------|----------|---------|------------|
| Met to G1      | 1.6 × 10⁷ (100)* | 4.8 × 10⁷ (100) | 2.5 × 10⁷ (100) | 1.8 × 10⁷ (100) |
| Mid G1         | 1.0 × 10⁷ (69.4) | 2.7 × 10⁷ (56.2) | 1.6 × 10⁷ (64.0) | 1.7 × 10⁷ (95)   |
| Late G1        | 1.0 × 10⁷ (68)  | 2.5 × 10⁷ (52)   | 1.5 × 10⁷ (60)   | 1.6 × 10⁷ (93)   |

Incorporation of lipid precursors in met to G1 cells, mid G1 cells, and late G1 cells. HeLa cells were synchronized in metaphase (90%), in mid G1 (4 h), and in late G1 (6.5 h), and exposed to radioactive glycerol, choline, methionine, or orthophosphate for 90 min as described in Materials and Methods. *methyl-³H* methionine labeling was done in the presence of cycloheximide. Inhibitor and radioactivity were added simultaneously in metaphase populations; in mid and late G1 populations, inhibitor was added 15 min prior to the radioactivity. Chloroform/methanol-soluble and TCA-water-soluble fractions were prepared from each population, and the amount of radioactivity or radioactivity was determined as described in Materials and Methods. Values for the chloroform/methanol-soluble fraction are presented (see text) and represent the average of three determinations. For comparison, the data obtained with the metaphase population (met to G1) have been given a value of 100%.

* Values in parenthesis are percentages.
thionine labeling, ~74, 16, and 9% of the label were recovered in PC, LPC, and SPH, respectively. Taken together, these results suggested that mitotic cells were more active relatively in the synthesis of PC by the CDP-choline plus diacylglycerol pathway.

Further Analysis of PC Synthesis

PC was one of the most abundant lipid classes in the membranes of cultured mammalian cells (26, 30), and it was possible to locate this component on thin-layer plates without adding carrier lipid to the extract. This permitted a direct determination of net synthesis, as well as a limited analysis of the species of PC synthesized.

When cells were pulsed with either radioactive phosphate or glycerol, and the radioactivity and phospholipid phosphate in the PC eluted from the thin-layer plate were determined, the specific activities (cpm/μmol phosphatidylcholine Pi) were over two- to threefold higher in material extracted from cells completing mitosis compared with late G1 cells (Table III). This PC material was further analyzed by chromatography on silver nitrate-impregnated silica gel, which separates PC species on the basis of the degree of saturation of the esterified fatty acids (Fig. 2). Rapidly migrating fractions contain the smallest number of double bonds whereas the slowly migrating fractions contain mixtures of PC containing fatty acids with multiple levels of unsaturation. When analyzed in this manner, PC synthesized in cells completing mitosis migrated rapidly as a marker species containing zero to two double bonds, whereas material synthesized in subsequent late G1 yielded both fast and slow material. The latter migrated heterogeneously as a mixture of both fast and slow material, as well as a slow fraction containing the smallest number of double bonds. This PC was further analyzed with late G1 cells (Table III).

Intracellular Site of Incorporation of PC in Late Mitotic Cells

Since late mitotic cells demonstrated a net increase in synthesis of PC, sections of whole cells were examined using EMARS to visualize the intracellular site of this incorporation. Essentially, populations of metaphase cells were suspended in choline-deficient medium containing high levels of tritiated choline, and by incubation, were permitted to progress to the late stages of mitosis. Then, as described in Materials and Methods, cells completed mitosis (met to G1), and late G1 cells were labeled with radioactive orthophosphate or glycerol as described in Table III. Chloroform/methanol-soluble extracts were prepared and the classes of phospholipids were separated by two-dimensional chromatography. PC was eluted from the silica gel with two chloroform washes, and radioactivity and inorganic phosphate were determined.
processed cells (as mentioned earlier). Initial experiments revealed that sections contained relatively small numbers of grains and that any one section did not always mirror the quantified results obtained from a large number of photographs. Presumably, this lack of uniform distribution represented domains of incorporation due to short labeling times and/or extensive structural rearrangements of ER in mitotic cells. Therefore both individual photographs and the quantification based on random sections through mitotic cells have been scored. Consistent with the previous micrographs, the majority of the grains were distributed over the cytoplasm, and careful examination revealed that they were at or near cytoplasmic memranous structures such as mitochondria and vesicles. This quantification also indicates significant incorporation at least by telephase in the vicinity of the NE. The latter totaled ~25% of the grains observed and, when the data were analyzed on the basis of relative area, the NE appeared to contain a greater proportion of the intracellular radioactivity compared with the cytoplasmic membrane.

**DISCUSSION**

Precise information concerning membrane lipid synthesis during cell division has been lacking. Thus, it has not been clear whether or not intrinsic changes in these lipids occurred during mitosis, and whether or not synthesis of new lipid components could be correlated with an observed ultrastructural event, such as NE reformation. We have studied the latter question concerning new synthesis with cells tightly synchronized in metaphase and with labeling times restricted to the time required for the metaphase cell to complete mitosis. This labeling time is significantly shorter than the half-life of the major phospholipids (for a review see, reference 26) and, thus, focuses attention on newly synthesized material.
FIGURE 3 Visualization of choline incorporation in late mitotic cells. A preparation of metaphase cells (92%) was suspended in medium containing [3H]choline at a concentration of 100 μCi/ml, as described in Materials and Methods, and incubation was continued for 40 min at 37°C. After fixation and embedding in EPON, gold-to-silver sections were mounted on grids and EMAR was performed using Ilford L4 emulsion (see Materials and Methods). A is a section through a metaphase cell; B is a section through an anaphase cell; C is a section through a telophase cell; and D is higher magnification of a section through a telophase cell. Details are described in text. Arrows indicate the location of silver grains. In D, only NE-associated grains are indicated. (A–C) × 10,625; (D) × 18,250.
Also, extracted components were analyzed by two-dimensional thin-layer chromatography that clearly separated 12 classes of phospholipids. With this detailed approach, it is clear that late mitotic cells, i.e., anaphase, telophase, as well as early G1 cells, were not only active in maintaining synthesis of phospholipids but also exhibited an increased net synthesis of PC that appeared to be oriented toward the more saturated species. Furthermore, visualization of this incorporation into individual mitotic cells revealed that a significant amount of newly synthesized PC could be detected in the NE coincident with its reformation in telophase, prior to the formation of progeny G1 cells. Whether or not this coincidence reflects a causal relationship between this synthesis pattern and NE reformation is unknown at present. Our data are consistent with this idea, but they do not rule out alternative explanations. For example, it is conceivable that at any time in the life cycle of the cell a large fraction of the newly synthesized PC passes through the NE. Presumably, this incorporation would represent transport from the ER, the site of PC synthesis. Also, other membranous systems such as the ER and plasma membrane are undergoing major ultrastructural rearrangements during late mitosis and thus, any speculations based on our data could apply equally well to these membranes.

Regardless of which membrane system(s) may be affected, implicit in our data is the concept that an altered pattern of PC synthesis reflects a membrane compositional change(s). It follows then that this could be an integral part of the mitotic process. Several characteristics of PC and its metabolism are compatible with this idea. First of all, PC itself is thought to impart membrane ultrastructure (for a review see reference 9) and thus, may be of major importance in re-establishing, for example, NE integrity. The putative half-life of PC is often relatively short and considerably less than a cell generation time (27). For example, in cultured baby hamster kidney cells labeled with glycerol, it has been estimated that PC has a half-life of 2–4 h, that phosphatidylethanolamine (PE) has a half-life of 4–8 h, and that phosphatidylinositol (PI) has a half-life of 15 h (13). Therefore, significant changes in composition could be introduced via PC within the immediate time limits of the division process. In addition, changes in fluidity may be important for membrane rearrangements at mitosis, and several ideas focus on PC with regard to this property. Among these are compositional changes involving the ratio of PC to PE (15), the ratio of PC to SPH (2), the ratio of unsaturated fatty acids to saturated fatty acids (6), and the ratio of long-chain to short-chain fatty acids (6). Presently, our data stress the competition between saturated and unsaturated fatty acids. In fact, it has been reported that PC synthesized from PE preferentially introduces higher polyunsaturated acids, whereas PC synthesized from CDP-choline and diacylglycerol preferentially introduces more saturated fatty acids (17, 26). It is this latter pathway that our data indicate was augmented in late mitosis.

It is becoming increasingly obvious that the mammalian cell is highly structured, and it has been suggested that structural reorganization is a controlling element in cellular function. With regard to this idea, cytoplasmic cytoskeletons and nuclear matrices are receiving much attention, and it has been suggested that the biochemical composition and the synthesis of components of such structural elements conceivably could play specific regulatory roles (12). It also is conceivable that membranous structural elements, such as an NE, could play regulatory roles in cell division and that their behavior is controlled via aspects of PC metabolism. In fact, the amount of PC synthesized is under the rate-limiting activity catalyzed by CTP-phosphocholine cytidylyltransferase (28, 29), a single enzyme in its biosynthetic pathway.

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