PHOSPHOLIPID METABOLISM IN THE MCF-7 CELL CYCLE: REGULATION OF PHOSPHATIDYLCHOLINE ACCUMULATION

BY

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A thesis
submitted to the Faculty of Graduate Studies
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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This thesis is dedicated

to

my wife and my parents

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LIST OF ABBREVIATIONS

Ab	antibody
AEBSF	aminoethylbenzenesulfonyl fluoride
Ala	alanine
AMP	adenosine 5'-monophosphate
ATP	adenosine 5'-triphosphate
BrdU	bromo-deoxyuridine
BSA	bovine serum albumin
CAK	Cdk-activating kinase
cAMP	cyclic adenosine 3', 5'-monophosphate
CdK	cyclin-dependent kinase
CDP	cytosine 5'-diphosphate
CDP-Cho	cytidine diphosphocholine
CDP-Etn	cytidine diphosphoethanolamine
Cho	choline
CPEC	cyclopentenylcytosine
$cPLA_2$	Ca^{2+}-dependent cytosolic phospholipase A_2
CPT	CDP-choline:diacylglycerol cholinephosphotransferase
CSF	colony-stimulating factor
CTP	cytosine 5'-triphosphate
DAG	diacylglycerol

dCDP	deoxyribocytidine diphosphate
dCTP	deoxyribocytidine triphosphate
dNTP	deoxynucleotide triphosphate
DDW	distilled deionized water
DHU	double hydroxyurea
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
EGTA	ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid
EPT	CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase
Etn	ethanolamine
FA	fatty acid
FA-CoA	fatty acyl-coenzyme A
FACS	fluorescence-activated cell sorter
FBS	fetal bovine serum
GPC	glycerophosphocholine
GPL	glycerophospholipid
GTP	guanosine 5'-triphosphate
Hank's	Hank's balance saline solution
HU	hydroxyurea

IP_3	inositol-1,4,5-trisphosphate
$iPLA_2$	Ca^{2+}-independent cytosolic phospholipase A_2
kDa	kilodaltons
K_m	Michaelis-Menten coefficient
lysoPC	lysophosphatidylcholine
lysoPE	lysophosphatidylethanolamine
lysoPG	lysophosphatidylglycerol
lysoPI	lysophosphatidylinositol
lysoPL	lysophospholipid
lysoPLA	lysophospholipase A
lysoPS	lysophosphatidylserine
MAG	monoacylglycerol
MAPK	mitogen-activated protein kinase
mM	millimolar
mRNA	messenger ribonucleic acid
NP-40	nonidet P-40
P-Cho	phosphocholine
P-Etn	phophoethanolamine
PA	phosphatidic acid
PAF	platelet-activating factor
PBS	phosphate buffer saline solution
PC	phosphatidylcholine

PC-PLC	phosphatidylcholine-specific PLC
PCCT	CTP:phosphocholine cytidylytransferase
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PECT	CTP:phosphoethanolamine cytidylyltransferase
PG	phosphatidylglycerol
PI	phosphatidylinositol
PI-PLC	phosphoinositide-specific PLC
PIP	phosphatidylinositol-4-phosphate
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKC	protein kinase C
PLA_1 (or A_2)	phospholipase A_1 (or A_2)
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PP1	type 1 protein phosphatase
PP2A	type 2A protein phosphatase
Pro	proline
PS	phosphatidylserine
Rb	retinoblastoma protein
RNase A	Ribonuclease A
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Ser	serine
SM	sphingomyelin
$sPLA_2$	secretory phospholipase A_2
TAG	triacylglycerol
TCA	trichloroacetic acid
Thr	threonine
TLC	thin-layer chromatography
Tris	tris-hydroxymethyl aminomethane
Tyr	tyrosine
UTP	uridine triphosphate
μCi	microcurie

ABSTRACT

Cells reproduce by duplicating their components, doubling their size and dividing in two. Since phospholipids are major components of cell membranes, cells must double their phospholipid mass so that the daughter cells formed have the same membrane composition as the parent. However, the mode of phospholipid accumulation and how this is regulated within the cell cycle remained largely unknown. The objective of this project is to investigate how membrane phospholipid accumulation is coordinated and regulated in the cell cycle, with special emphasis on the accumulation of phosphatidylcholine (PC).

Two synchronization protocols were developed that synchronized approximately 90% of the cells at specific points in the cell cycle. The double hydroxyurea treatment synchronized MCF-7 cells at the G1/S boundary while the combination of hydroxyurea and nocodazole blocked the cells at mitosis. Synchronized cells at different stages of the cell cycle were obtained by harvesting cells at various times after removal of the cell cycle blocking agents. Measurement of phospholipid mass at different stages of the cell cycle revealed that the contents of PC, phosphatidylethanolamine (PE) and phosphatidylinositol (PI) declined during S phase followed by a net accumulation in G2/M phase. The relative composition of PC, PE and PI remained unchanged throughout the cell cycle, suggesting that there might be coordinated metabolism of these lipids in the cell cycle. Further studies demonstrated that the rate of incorporation of [^{3}H]choline into PC was low in early S phase, increased from late S and throughout G2/M. The fluxes in PC synthesis correlated with changes in the activities of CTP:phosphocholine cytidylyltransferase (PCCT),

cholinephosphotransferase (CPT) and the levels of membrane-associated PCCT. The results suggest that PC synthesis contributes to the fluctuation in PC levels observed during the MCF-7 cell cycle. On the other hand, there was little change in the rate of incorporation of [^{14}C]ethanolamine into PE during S and G2/M phases. The activity of CTP:phospho-ethanolamine cytidylyltransferase (PECT), the rate limiting enzyme in PE synthesis, showed little variation in the cell cycle, suggesting that the rate of PE catabolism is the dominant factor in regulating PE levels during the cell cycle. No evidence was obtained to implicate changes in cellular CTP levels in the cell cycle regulation of phospholipid synthesis. Studies on PC catabolism revealed that the rate of PC catabolism was enhanced during S phase but terminated in the G2/M phase.

Taken together, our studies demonstrated that the decrease in PC content during S phase resulted from enhanced PC catabolism, while PC accumulation in G2/M was attributed to both enhanced PC biosynthesis and cessation of PC catabolism.

1 INTRODUCTION

1.1 The biological membrane

1.1.1 Overview of biological membranes

Biological membranes are very crucial to the life of a cell. They comprise the plasma membrane which forms a closed compartment around the cell to separate it from its environment and thus, permit cellular individuality, and intracellular membranes which form many of the morphologically distinguishable structures (organelles) within the cell, such as mitochondria, endoplasmic reticulum, Golgi apparatus, lysosomes, peroxisomes, and nucleus. These organelles perform various functions essential to cell viability.

1.1.2 The structure and function of biological membranes

1.1.2.1 The structure of biological membranes

All biological membranes have a common basic structure. As summarized in the Singer and Nicolson fluid mosaic model (Figure 1), biological membranes consist of a lipid bilayer, proteins and carbohydrate moieties associated with the lipid and proteins (Singer and Nicolson, 1972). Biological membranes from various organelles differ in the ratio of protein to lipid (Figure 2). The lipid bilayer is composed primarily of phospholipids and cholesterol. Embedded in the lipid bilayer are proteins with a variety of important cellular functions, such as receptors, transporters and enzymes. The lipid and protein molecules are held together mainly by noncovalent interactions.

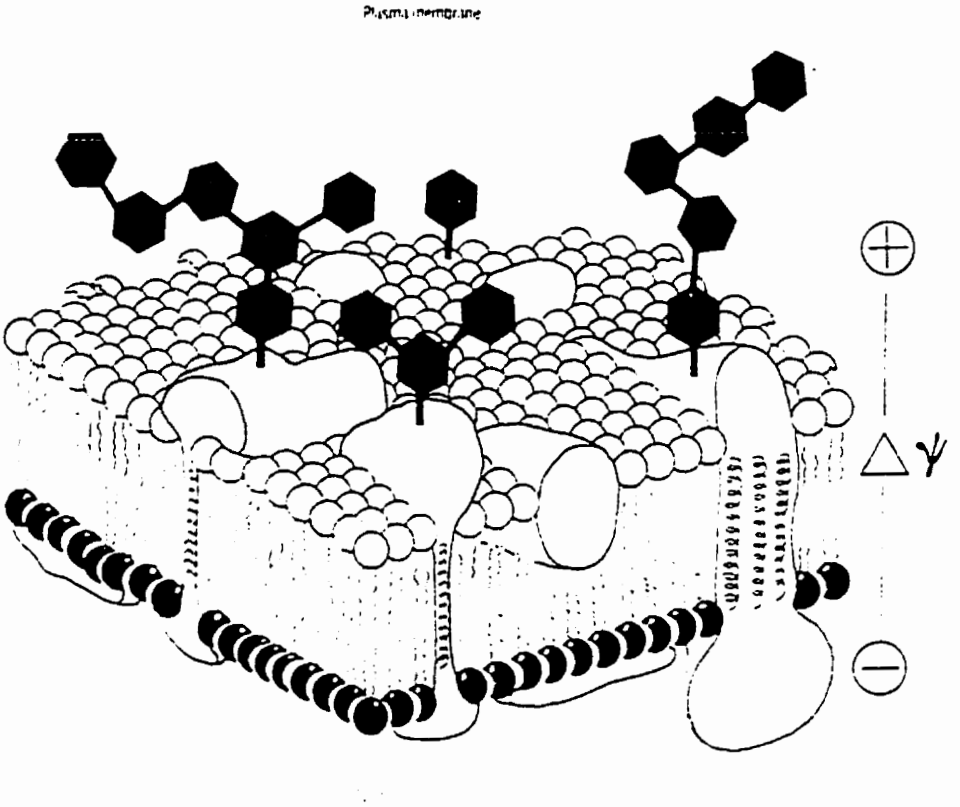


Figure 1. **The fluid mosaic model of the eukaryotic plasma membrane** depicting the lipid bilayer, proteins, and carbohydrate moities associated with both lipids and proteins. The membrane potential $\Delta\Psi$ arises from transbilayer electrochemical gradients of various ions. Adapted from Cullis and Hope (1991).

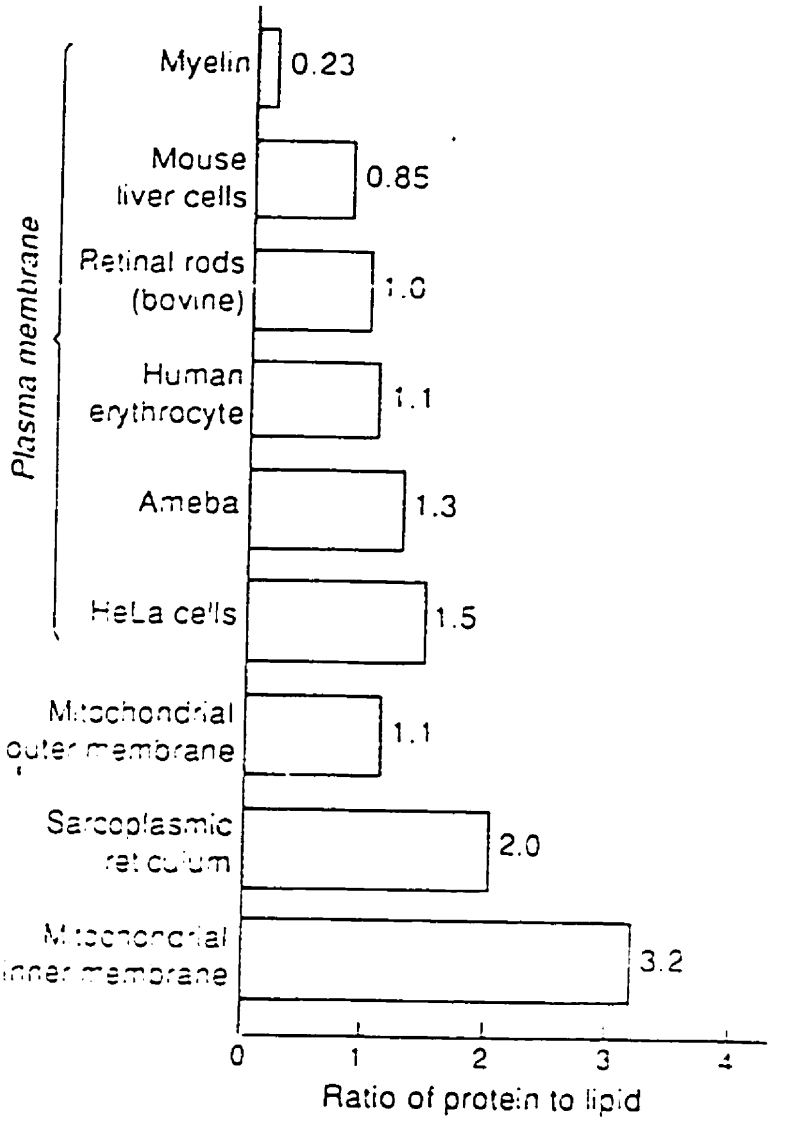


Figure 2. **Ratio of protein to lipid in different membranes.** Adapted from Murray *et al.* (1996).

1.1.2.2 The functions of biological membranes

Cell membranes are dynamic fluid-like structures that allow rapid lateral diffusion of the lipid and protein molecules in the plane of the membrane (Cullis and Hope, 1991). The lipid bilayer provides the basic structure of the membrane and serves as a barrier which is selectively permeable to various molecules. This allows cells to maintain differences in composition between the inside and outside of the cell, as well as between organelles and the cytosol. The selective permeabilities are provided by various channels and ions pumps. Plasma membranes also exchange material with the extracellular environment through exocytosis and endocytosis and there are special areas of membrane structures, the gap junctions, through which adjacent cells exchange material. In addition, the membranes are considered as a reservoir from which cells generate intracellular and intercellular messengers.

The membrane proteins embedded in the lipid bilayer mediate many important cellular functions, this includes transporting specific molecules across the membrane, catalyzing membrane-associated reactions, serving as structural links that connect the membrane to the cytoskeleton and/or to either the extracellular matrix of an adjacent cell, serving as receptors to detect and transduce signals into the cell's environment.

1.1.3 Membrane lipids

Lipid molecules are insoluble in water but dissolve readily in organic solvents. They make up about 50% of the mass of most animal cell membranes. Membrane lipids are amphipathic molecules, with a hydrophilic head and a hydrophobic tail. The major lipids in mammalian membranes are phospholipids, glycosphingolipids and cholesterol.

1.1.3.1 Phospholipids

Glycerophospholipid (GPL) and sphingomyelin (SM) are two major phospholipids present in biological membranes. GPLs are the more common and have a glycerol backbone to which two fatty acids are attached by an ester linkage at the *sn*-1 and *sn*-2 positions and a phosphorylated alcohol (base) at the *sn*-3 position (Figure 3). The two fatty acids, which provide the hydrophobicity in the molecule, can differ in length and normally contain 14 to 24 carbons. Saturated fatty acids are usually esterified at the *sn*-1 position whereas unsaturated fatty acids are commonly found at the *sn*-2 position of the glycerol backbone. Each double bond creates a small kink in the fatty acid and thus influences the ability of phospholipid molecules to pack against one another. The saturation of the fatty acid tails, therefore, can modulate the fluidity of the membrane (Quinn, 1981). The simplest GPL is lysophosphatidic acid (lysoPA), which is 1-acylglycerol-3-phosphate. Acylation of lysoPA yields phosphatidic acid (1,2-diacylglycerol-3-phosphate), a key intermediate in the formation of all other GPLs. In other GPLs, the 3-phosphate in phosphatidic acid is esterified to a base, such as choline, ethanolamine, serine, glycerol, or inositol (Table 1).

Sphingomyelins have a sphingosine backbone rather than glycerol. A fatty acid is attached by an amide linkage to the amino group of sphingosine. The primary hydroxyl group of sphingosine is esterified to phosphorylcholine (Figure 4). Sphingomyelins are prominent in the myelin sheath.

1.1.3.2 Glycosphingolipids

Glycosphingolipids are found in eukaryotic cell plasma membrane and generally

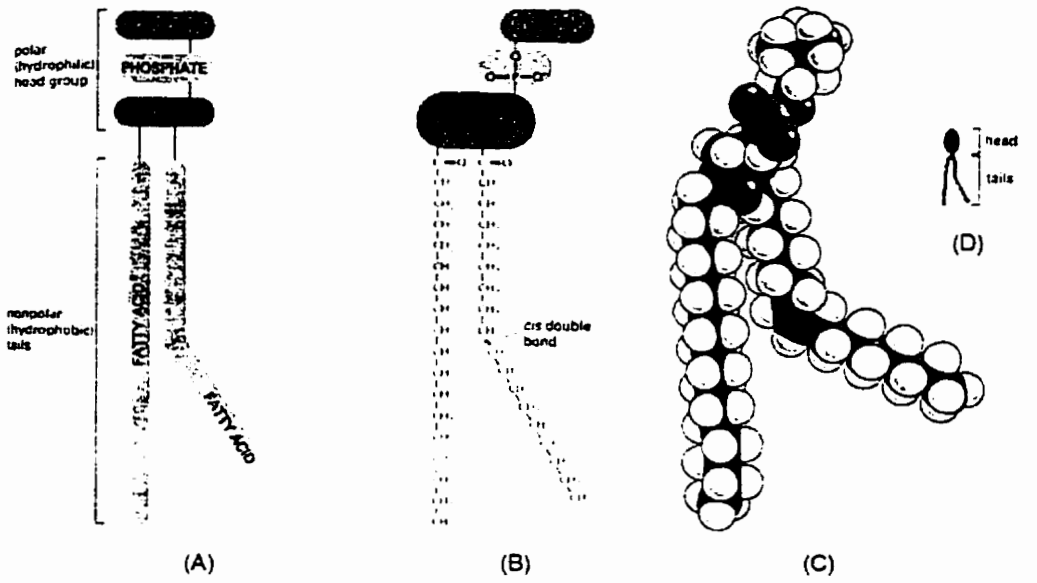
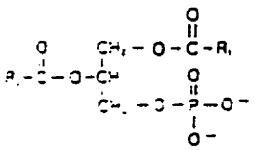
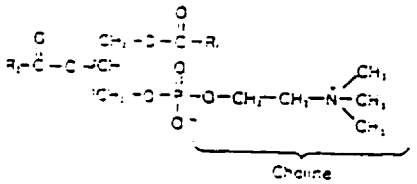
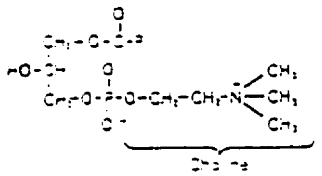
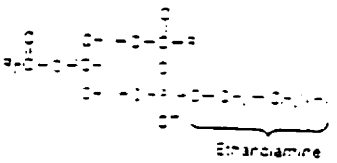
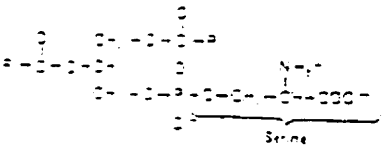
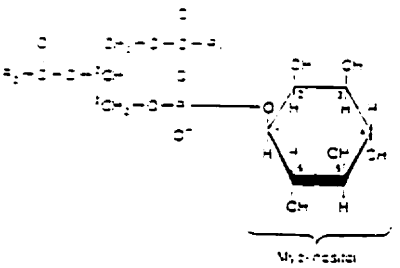
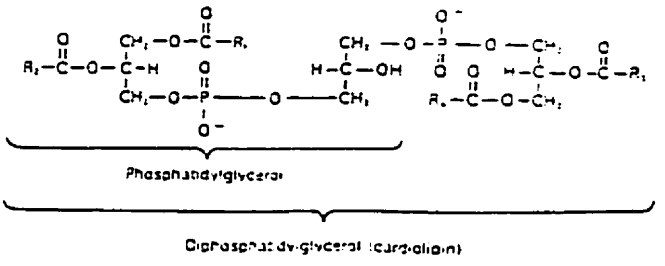


Figure 3. **The basic structure of a phospholipid molecule**. The major phospholipid, phosphatidylcholine is represented schematically (A), in formula (B), as a space-filling model (C), and as a symbol (D). Adapted from Alberts *et al.*, (1994a) Membrane structure. In *Molecular Biology of the Cell*, 3rd Ed., Garland Publishing, Inc., New York, pp.479.

Table 1. **Major classes of glycerolphospholipids**

Phosphatidic acid (PA)	
Phosphatidylcholine (PC)	
Lysophosphatidylcholine (lysoPC)	
Phosphatidylethanolamine (PE)	
Phosphatidylserine (PS)	
Phosphatidylinositol (PI)	
Phosphatidylglycerol (PG) and Cardiolipin	

constitute about 5% of the lipid molecules. They are sugar-containing lipids such as cerebrosides and gangliosides. Glycosphingolipids are derived from sphingosine. The cerebrosides and ganliosides differ from SM in the moiety attached to the primary hydroxyl group of sphingosine. In SM, a phosphocholine is attached to the hydroxyl group. A cerebroside contains a single hexose moiety, glucose or galactose attached to the primary alcohol of sphingosine (Figure 5), whereas a ganglioside contains a chain of three or more sugars at that site. Glycolipids are generally found on the extracellular leaflet of plasma membranes and function in cellular recognition and adhesion.

1.1.3.3 Cholesterol

Cholesterol, an important component of membranes, exists almost exclusively in the plasma membranes of mammalian cells. It is generally more abundant in the outer leaflet of the plasma membrane. The cholesterol molecules intercalate among the phospholipids and enhance the permeability-barrier properties of the lipid bilayer. Its rigid sterol ring interacts with the acyl chains of the phospholipids, limits their movement, and thus reduces membrane fluidity. Therefore, cholesterol can modulate membrane fluidity (Bloch,1991). Cholesterol is not only an important membrane constituent but also plays a vital role as a metabolic intermediate. It acts as a precursor for bile acids and the steroid hormones, such as glucocorticoids, aldosterone, oestrogens, progesterones, and androgens (Bloch,1991).

1.1.3.4 Membrane lipid diversity

As mentioned earlier, based on their different polar head groups, membrane GPLs

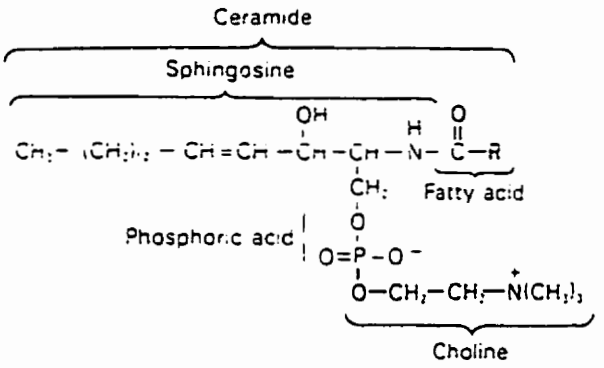


Figure 4. **Structure of sphingomyelin**

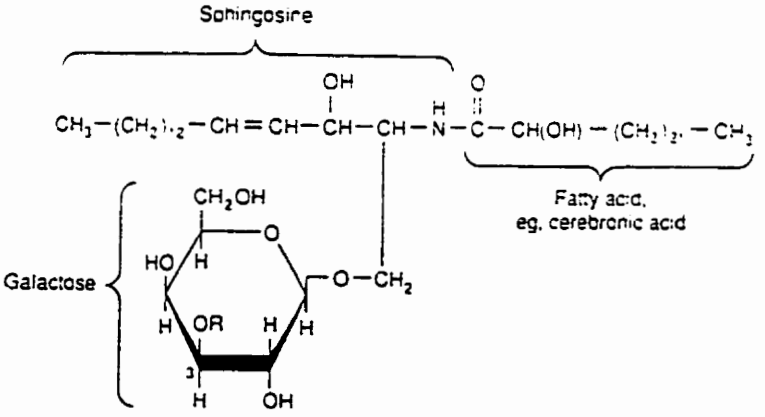


Figure 5. **Structure of galactosylcermide (R = H)**

can be classified into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), cardiolipin and phosphatidic acid (PA) (Table 1). PC and PE are the two major GPLs in mammalian cells. They constitute approximately 50% and 30% of total cellular GPLs respectively, while PI, PS , PG, cardiolipin and PA are the minor GPLs. Cardiolipin, which is mainly found in the inner membrane of mitochrondria, is synthesized by the condensation of two molecules of PG (Kent, 1995; Hatch, 1998).

The lipid composition of membrane can vary dramatically among different cells or organelles (Table 2). Significant differences in cholesterol content are also observed in different cellular membranes. Plasma membranes contain more than 20% of cholesterol, whereas the endoplasmic reticulum or the inner mitochondria membrane contain only small amounts of cholesterol. The fluidity of the membrane lipid bilayer can be modulated by the cholesterol content and the saturation of the fatty acids. The cholesterol distribution correlates well with the distribution of SM, which is relatively saturated. This suggests that cholesterol may play a 'fluidizing' role in membranes containing large amount of SM (Alberts *et al.*, 1994a).

Phospholipids contain a variety of molecular species defined by the fatty acid composition (Table 3). PE and PS contain more unsaturated fatty acids than the other phospholipids.

1.1.3.5 Asymmetry of the lipid bilayer

The inner and outer leaflets of membrane lipid bilayers exhibit different lipid

Table 2. **Lipid composition of different cell membranes**. Adapted from Alberts *et al.*, (1994) Membrane structure. In *Molecular Biology of the Cell*, 3rd Ed., Garland Publishing,

Approximate Lipid Compositions of Different Cell Membranes						
	Percentage of Total Lipid by Weight					
Lipid	Liver Plasma Membrane	Erythrocyte Plasma Membrane	Myelin	Mitochondrion (inner and outer membranes)	Endoplasmic Reticulum	*E. coli*
Cholesterol	17	23	22	3	6	0
Phosphatidyl-ethanolamine	7	18	15	35	17	70
Phosphatidylserine	4	7	9	2	5	trace
Phosphatidyl-choline	24	17	10	39	40	0
Sphingomyelin	19	18	8	0	5	0
Glycolipids	7	3	28	trace	trace	0
Others	22	13	8	21	27	30

Table 3. **Gas chromatographic analysis of the fatty acid chains in various human red cell phospholipids.** Adapted from Cullis and Hope (1991) Physical properties and functional roles of lipids in membranes. In *Biochemistry of lipids, lipoproteins and Membranes* (Vance, D.E., and Vance, J., eds.), pp. 5.

Chain length and unsaturation	Total phospholipids	Sphingomyelin	Phosphatidyl-choline	Phosphatidyl-ethanolamine	Phosphatidyl-serine
16:0*	20.1	23.6	31.2	12.9	2.7
18:0	17.0	5.7	11.8	11.5	37.6
18:1	13.3	+	18.9	13.1	8.1
18:2	9.6	–	22.8	7.1	3.1
20:0	–	1.9	–	–	–
20:3	1.3	–	1.9	1.5	2.6
22:0	1.9	9.5	1.9	1.5	2.6
20:4	12.6	1.4	6.7	23.7	24.2
23:0	–	2.0	–	–	–
24:0	2.7	22.8	–	–	–
22:4	3.1	–	–	7.5	4.2
24:1	4.8	24.0	–	–	–
22:5	2.0	–	–	4.3	3.4
22:6	4.2	–	2.1	8.2	10.1

The data are expressed as weight % of the total. + Denotes that the concentration did not exceed 1% of the total.

*This code indicates the number of carbon atoms in the chain and the number of double bonds.

composition (Op den Kamp, 1979). Studies have shown that the majority of amino-containing phospholipids, such as PE and PS, are located on the inner monolayer of plasma membrane lipid bilayer. The outer monolayer consists predominantly of PC, SM, and glycosphingolipid. Figure 6 summarizes the phospholipid distribution in the inner and outer leaflets of various membranes. A common feature of plasma membrane asymmetry is that the majority of phospholipids that exhibit a net negative charge at physiological pH are limited to the cytosolic half of the lipid bilayer

1.1.4 Membrane proteins

Although the basic structure of biological membranes is provided by the lipid bilayer, most of the specific functions are carried out by proteins. The amounts and types of proteins in different membranes are highly variable: in the myelin membrane, less than 25% of the membrane mass is protein. whereas in mitochondria and chloroplast membranes, approximately 75% is protein (Figure 2). About 50% of the mass of plasma membrane is protein with the remainder being lipid.

Different membrane proteins are associated with the membrane in different ways. As illustrated in Figure 7, some proteins extend through the lipid bilayer, with part of the protein on either side of the bilayer. These proteins are referred to as transmembrane proteins (Figure 7, ① and ②). Transmembrane proteins are amphipathic, having both hydrophobic and hydrophilic domains. Their hydrophobic domains interact with the hydrophobic tails of the lipid molecules in the interior of the lipid bilayer. Their hydrophilic regions are exposed to water on one or the other side of the membrane. Other membrane

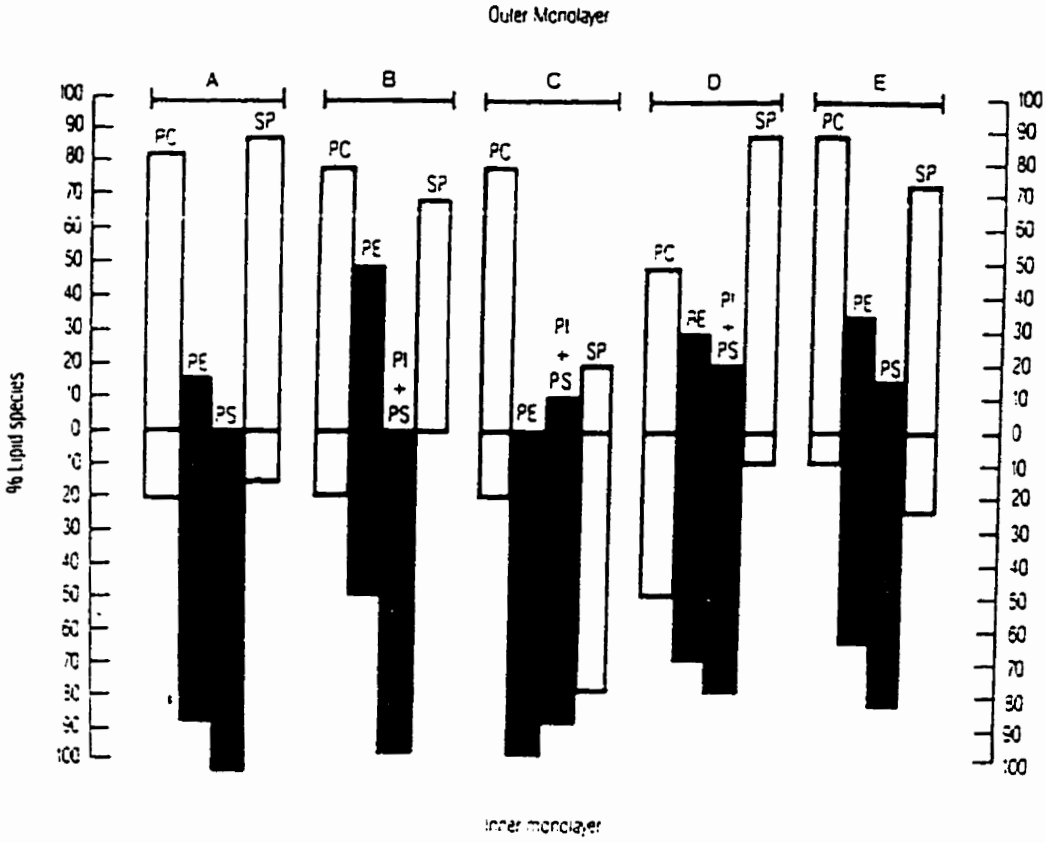


Figure 6. **Phospholipid asymmetry in various plasma membranes.** (A) Human erythrocyte membrane. (B) rat liver blood sinusoidal plasma membrane, (C) rat liver continuous plasma membrane, (D) pig platelet plasma membrane. (E) VSV envelope derived from hamster kidney BHK-21 cells. Adapted from Cullis and Hope (1991) Physical properties and functional roles of lipids in membranes. In *Biochemistry of lipids, lipoproteins and Membranes* (Vance, D.E., and Vance, J., eds), pp. 7.

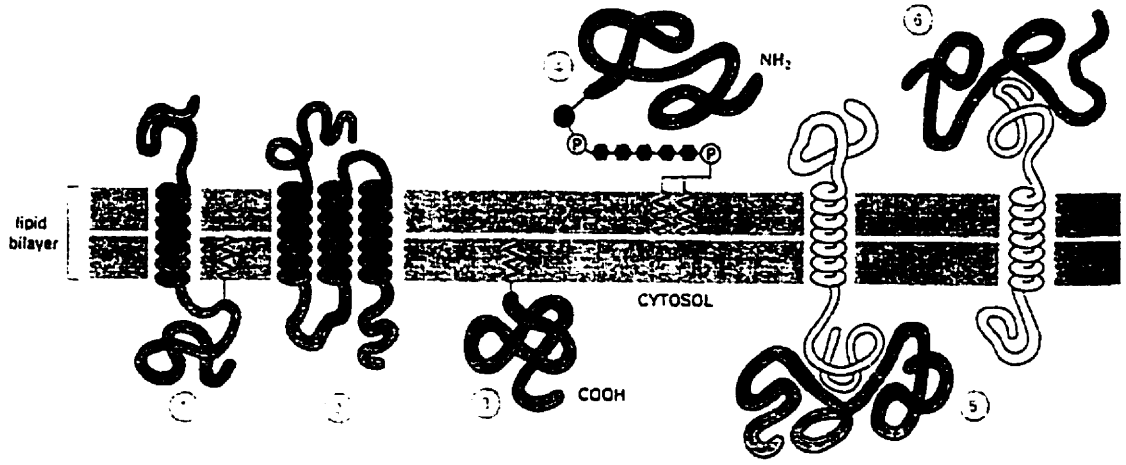


Figure 7. **Membrane protein association with the lipid bilayer.** Transmembrane protein (① and ②); peripheral membrane proteins linked by prenyl groups (③ and ④) or by noncovalent interaction (⑤ and ⑥). Adapted from Alberts *et al.*, (1994a) Membrane structure. In *Molecular Biology of the Cell*, 3rd Ed., Garland Publishing, Inc., New York, pp.486.

proteins are located entirely on one side of the membrane. Some are associated with the lipid bilayer through a covalent attachment with fatty acid chains or a prenyl groups (Figure 7, ③ and ④). Other membrane proteins are bound to the surfaces of the membrane by noncovalent interactions with lipids or other membrane proteins. These proteins are referred as peripheral membrane proteins (Figure 7, ⑤ and ⑥).

Like the lipid molecules in the lipid bilayer, many membrane proteins are able to diffuse rapidly in the plane of the membrane. In the plasma membrane of all eukaryotic cells, most of the membrane proteins exposed on the cell surface are covalently attached to oligosaccharide chains. This sugar coating helps to protect the cell surface from mechanical and chemical damage (Alberts *et al.*, 1994a).

1.1.5 Membrane carbohydrates

Membrane carbohydrates are a minor component of plasma membrane. They are exclusively located in the outer leaflet of the plasma membrane lipid bilayer (Figure 1). They exist in the form of glycolipids and glycoproteins. Membrane carbohydrates have important functions in cell-cell adhesion and recognition. In addition, they can also modulate receptor activity, antigenic specificity, extracellular interactions, and growth regulation (Alberts *et al.*, 1994b; Sweely, 1991).

1.2 Phospholipid biosynthesis

1.2.1 Biosynthesis of phosphatidic acid, diacylglycerol and CDP-diacylglycerol

The biosynthetic pathway of phospholipids and triacylglycerol in mammalian cells

is depicted in Figure 8. Phosphatidic acid is synthesized by sequential acylation of glycerol-3-phosphate catalyzed by glycerophosphate acyltransferase and 1-acylglycerol-3-phosphate acyltransferase. Phosphatidic acid is a key intermediate in the formation of all other GPLs. After the synthesis of phosphatidic acid, the biosynthetic pathway of glycerophospholipids is separated into two branches in which two intermediates, diacylglycerol and CDP-diacylglycerol, are formed (Figure 8). Diacylglycerol, synthesized via phosphatidic acid phosphohydrolase, is the key precursor for PC, PE, and triacylglycerols biosynthesis. Whereas CDP-diacylglycerol, formed by CDP-diacylglycerol synthase, is used for the biosynthesis of PI, PG, cardiolipin, and in yeast, PS (Figure 8). Phosphatidic acid phosphohydrolase and CDP-diacylglycerol synthase are of interest because of their roles in controlling the flux into the two branches. In addition, there has been a great deal of interest in phosphatidic acid phosphohydrolase because both its substrate (PA) and product (DAG) can act as second messengers (Kent, 1995). The conversion from one to the other is likely to be a site of control.

1.2.2 Phosphatidylcholine biosynthesis

1.2.2.1 Introduction

Pathways for the synthesis of PC in mammalian cells have been established. There are two pathways for PC biosynthesis, one by methylation of PE and the other is the *de novo* biosynthetic pathway *via* CDP-choline discovered by Kennedy (Kennedy and Weiss, 1956). The former pathway predominates in yeast, whereas in mammals, the CDP-choline pathway is the predominant pathway for PC biosynthesis in almost all tissues. Liver is the only tissue

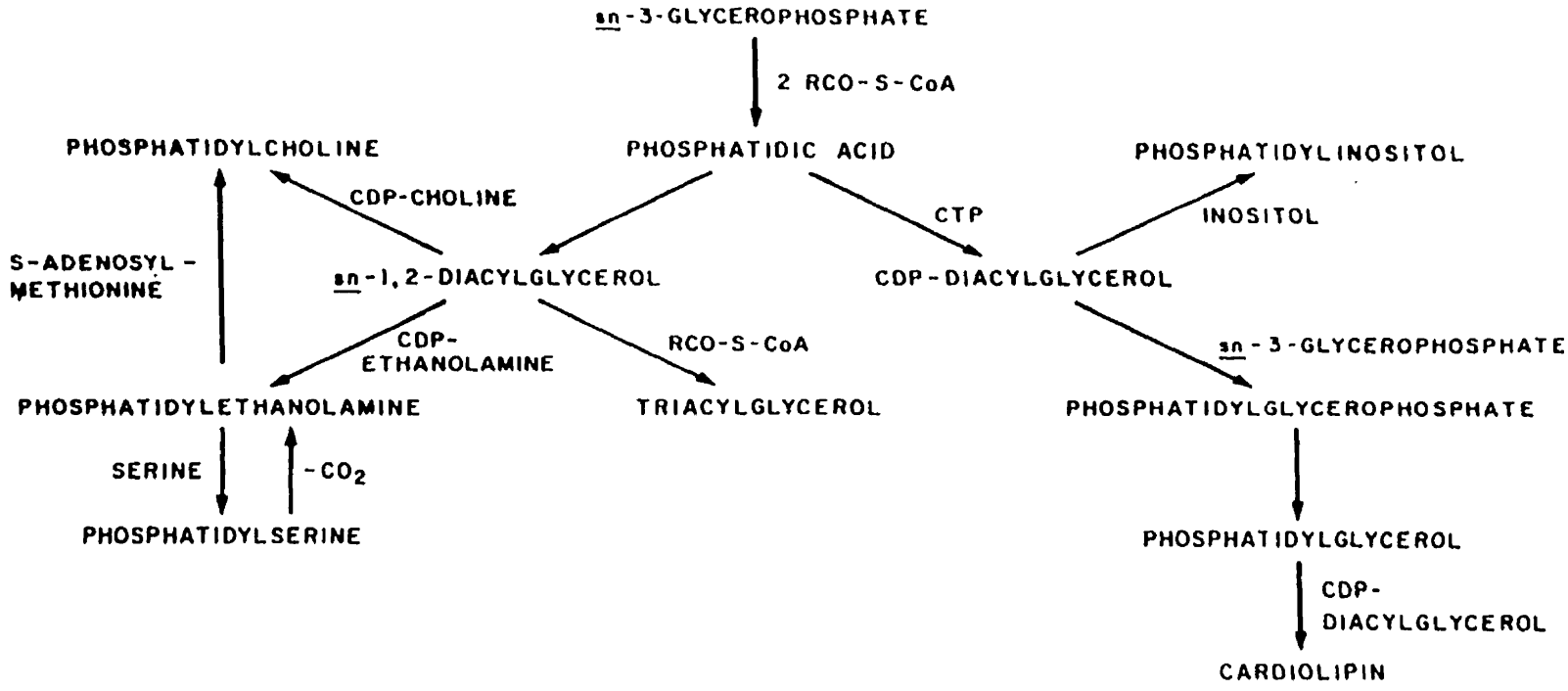


Figure 8. **Biosynthesis of phospholipids and triacylglycerol in animal tissues.** Adapted from **Vance,** (1989) phosphatidylcholine metabolism (Vance, D.E., ed.) CRC Press Inc., pp. 5.

in which a significant amount of PC is made via PE methylation. In addition, PC can also be derived from reacylation of lysophosphatidylcholine (lysoPC) (see section 1.2.2.3) and base-exchange of free choline with other preexisting GPLs. However, base-exchange represents only a minor pathway for the synthesis of most phospholipids except PS (Kanfer, 1980; Kanfer, 1989). It has been proposed that the base-exchange mechanism may play an important role in replenishing PC after substantial and prolonged breakdown of PC without immediate expenditure of energy (Kanfer, 1989).

1.2.2.2 PC biosynthesis *via* the CDP-choline pathway

The *de novo* pathway (CDP-choline pathway) is quantitatively the major pathway for PC biosynthesis (Kent, 1995; Choy *et al.*, 1997). In the CDP-choline pathway, choline is phosphorylated into phosphocholine which is converted to CDP-choline followed by condensation with diacylglycerol to form PC (Figure 9). Diacyglycerol is derived from hydrolysis of phosphatidic acid (section 1.2.1). The phosphorylation of choline is catalyzed by choline kinase which exists in multiple molecular forms in the cytosol (Ishidate, 1997). Choline kinase has been purified to homogeneity from a variety of tissues and the subunit molecular weights of the purified enzyme are reported to be 42 kDa for kidney (Ishidate *et al.*,1984), and 47 kDa for both liver (Porter and Kent, 1990) and brain (Uchida and Yamashita, 1990). The second step of the pathway is the formation of CDP-choline. This step has been recognized to be the rate-limiting step and is catalyzed by CTP:phosphocholine cytidylytransferase (PCCT) (Kent, 1997). PCCT has been purified to homogeneity from rat liver (Weinhold *et al.*, 1986; Feldman and Weinhold, 1987) and is a homodimer with a

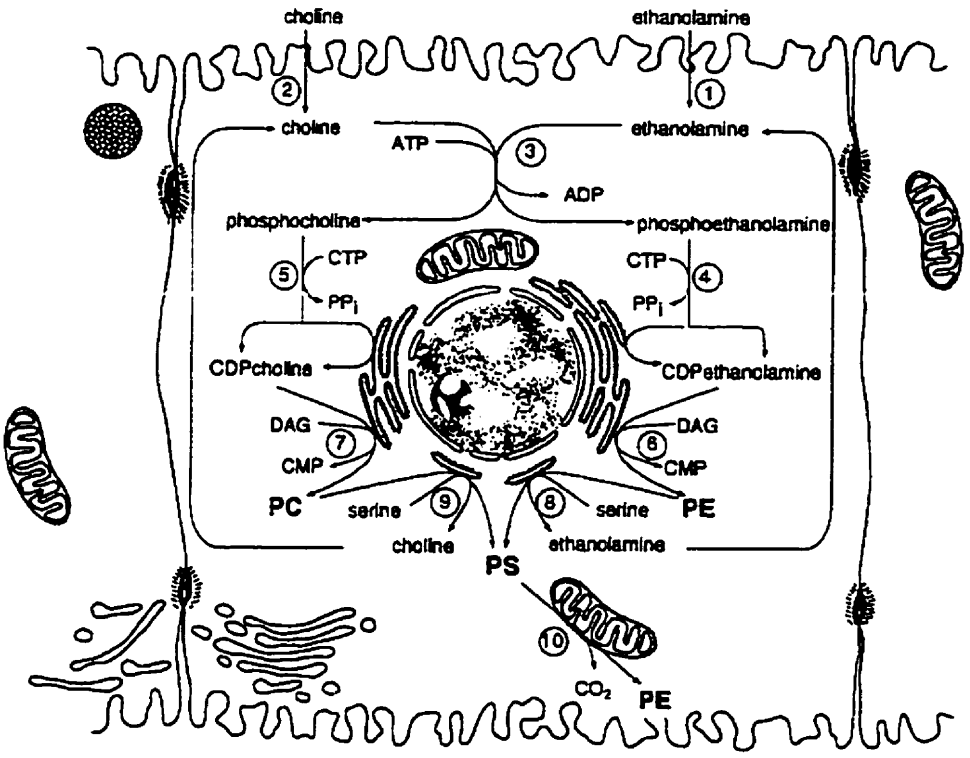


Figure 9. **Biosynthetic pathways of PC and PE.** The CDP-ethanolamine pathway comprises the reactions 3, 4, and 6; the CDP-choline pathway comprises the reactions 3, 5 and 7. PS is synthesized by base-exchange from either PE (reaction 8) or PC (reaction 9); PS can be converted to PE by decarboxylation (reaction 10). Cellular uptake of ethanolamine and choline is represented as 1 and 2. Enzymes: 3: choline/ethanolamine kinase; 4: CTP:phosphoethanolamine cytidylyltransferase; 5: CTP: phoshocholine cytidylytransferase; 6: ethanolaminephosphotransferase; 7: cholinephosphotransferase; 8: phosphatidylserine synthase II; 9: phosphatidylserine synthase I; 10: phosphatidylserine decarboxylase. Adapted from Vermeulen *et al.*, (1997) The CDP-Ethanolamine pathway in mammalian cells. In *Advances in Lipobiology*, **2**, 287-322.

subunit molecular weight of 42 kd (Weinhold *et.al.*, 1989; Cornell,1989a). A new isoform of human PCCT has recently been cloned and characterized (Lykidis, 1998). Regulation of PCCT will be discussed later (section 1.3). CDP-choline:diacylglycerol cholinephosphotransferase (CPT) catalyzes the final step of the CDP-choline pathway to form PC. CPT is located on the cytoplasmic side of the endoplasmic reticulum (McMaster and Bell, 1997a).

1.2.2.3 PC biosynthesis from lysophosphatidylcholine

In mammalian tissues, the majority of PC is synthesized *de novo via* the CDP-choline pathway. The last step of CDP-choline pathway, catalyzed by 1,2-diacylglycerol cholinephosphotransferase, has some specificity for certain molecular species of 1,2-diacylglycerol (Cornell, 1989b). However, gas chromatographic analysis reveals that there are hundreds of different molecular species of phospholipids in mammalian cells. It is clear that mechanisms must exist for remodelling the acyl content of phospholipids. The selection of the appropriate acyl groups in phospholipid is believed to take place after its synthesis through deacylation-reacylation which is also referred to as phospholipid remodelling (Choy and Arthur,1989)

Deacylation-reacylation has been generally accepted as the preferred mechanism for modulating the fatty acid distribution in phospholipids (Choy and Arthur,1989). Figure 10 depicts the deacylation-reacylation pathway of PC, in which PC is deacylated to lysoPC by phospholipase A_1 or A_2. The lysoPC formed can be further degraded to glycerophosphocholine (GPC) by lysophospholipases or reacylated back to PC by lysoPC:acyl CoA acyltransferase. The acyl-CoA derivative required for the reaction is formed from a

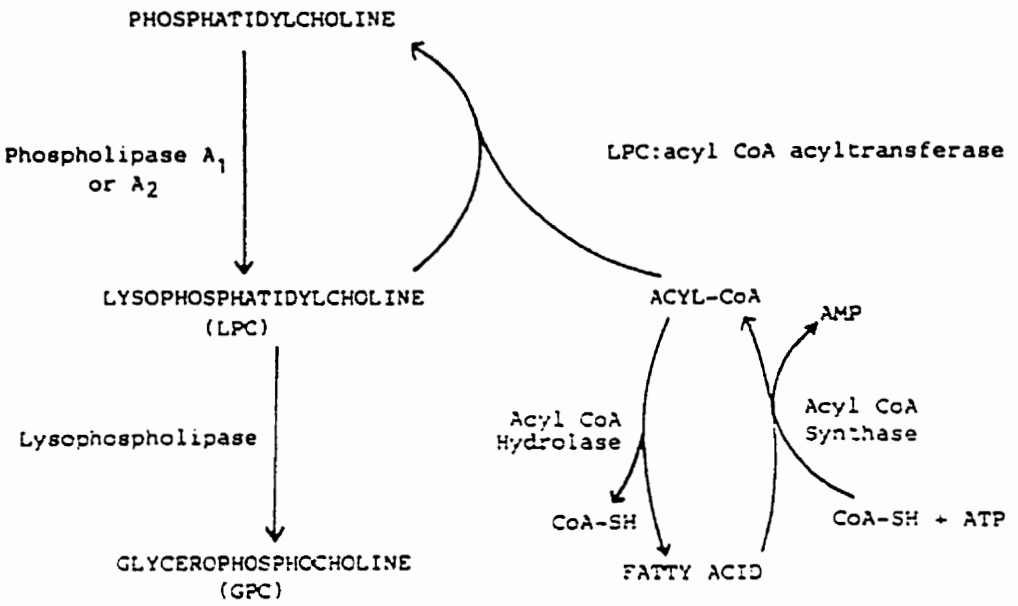


Figure 10. **The deacylation-reacylation pathway of phosphatidylcholine.** Adapted from Choy and Arthur (1989) Phosphatidylcholine biosynthesis from lysophosphatidylcholine. In *phosphatidylcholine metabolism* (Vance, D.E.., ed.) CRC Press Inc., pp. 88-101.

variety of fatty acids by the action of acyl-CoA synthetase (Choy and Arthur,1989). LysoPC has detergent properties and is cytolytic at high concentrations (Weltzien 1979). Thus, apart from its role in modulating the molecular composition of PC, the synthesis of PC from lysoPC also plays an important role to reduce the levels of this cytolytic molecule. Acylation processes may also serve to control the levels of free fatty acids (Irvine, 1982).

1.2.3 PE biosynthesis

Three pathways exit for the production of PE in mammalian cells. PE can be synthesized by base-exchange of free ethanolamine with preexisting phospholipids, by decarboxylation of PS or by the CDP-ethanolamine pathway (Vermeulen *et al.*, 1997). At physiological ethanolamine concentration, only 8-9% of the ethanolamine incorporation into PE was attributed to the direct base-exchange reaction. This contribution rose to 30-40% when ethanolamine concentration was raised twenty-fold (Sundler *et al.*, 1974).

The CDP-ethanolamine pathway, which is analogous to the CDP-choline pathway, is the major route for PE biosynthesis in most mammalian cells (Vermeulen *et al.*, 1997). However, decarboxylation of PS has been recognized as an important pathway for PE biosynthesis in prokaryotes and some eukaryotic cells (Voelker, 1984). In the CDP-ethanolamine pathway, ethanolamine is phosphorylated to phosphoethanolamine which is converted into CDP-ethanolamine and finally to PE (Figure 9). The rate limiting step of this pathway appears to be the conversion of phosphoethanolamine to CDP-ethanolamine but the phosphorylation of ethanolamine can also become rate-limiting under certain conditions (Vermeulen *et al.*, 1997; Vance 1991). Ethanolamine kinase and choline kinase appear to

be the same enzyme with different active sites (Ishidate, 1997). The conversion of phosphoethanolamine to CDP-ethanolamine is catalyzed by CTP:phosphoethanolamine cytidylyltransferase (PECT) (Bladergroen and Van Golde, 1997). Although PECT is generally considered as the rate limiting regulatory enzyme for PE biosynthesis, only limited information is available on its regulation. The enzyme was believed to reside exclusively in the cytosol and exists as a dimer with a subunit molecular weight of 49.6 kDa. However, Vermeulen *et al* noted that purified PECT had a high content of hydrophobic amino acids, suggesting that the enzyme might be associated with cellular structures (Vermeulen *et al.*, 1993). Subsequent immunoelectron microscopy studies using an affinity-purified antibody against PECT showed that the enzyme was not randomly distributed in hepatocytes (Van Hellemond *et al.*, 1994). The majority of PECT label was found in areas that contained cisternae of the endoplasmic reticulum. Within these areas, the PECT-label showed a bimodal distribution between the cisternae of the endoplasmic reticulum and the cytosol, suggesting that PECT could be regulated in a similar fashion to PCCT. Other cellular organelles, including nuclei, mitochondria, plasma membranes and Golgi, were only marginally labelled. The conversion of CDP-ethanolamine to PE is catalyzed by CDP-ethanolamine:diacylglycerol ethanolaminephosphotransferase (EPT). This enzyme is located in the endoplasmic reticulum (McMaster and Bell, 1997b).

1.2.4 Inositol phospholipid biosynthesis

Inositol lipids represent less than 10% of the total GPLs. Phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2), the preferred substrate of PI-phospholipase C, accounts for about

1-3% of the inositol lipids (Creba *et al.*, 1983). Phosphatidylinositol is synthesized by the condensation of CDP-diacylglycerol and myo-inositol catalyzed by phosphatidylinositol synthase. CDP-diacylglycerol, the precursor for PI, PG, and cardiolipin biosynthesis, is formed from the transfer of a cytidine diphosphate moiety from CTP to phosphatidic acid, a reaction catalyzed by CDP-diacylglycerol synthase (section 1.2.1). PI- 4,5-P_2 is formed by sequential phosphorylation of PI by PI 4-kinase and PI-4-P 5-kinase. It has been established that agonist-stimulated cell activation can trigger a phosphatidylinositol cycle, in which PI-4,5-P_2 is degraded by PI-phospholipase C into diacylglycerol and inositol-1,4,5-triphosphate (IP_3) (Alberts *et al.*, 1994c; Weber *et al.*, 1996). The diacylglycerol produced acts as a second messenger to activate protein kinase C, while the other product, IP_3, causes Ca^{2+} release from internal stores. In addition, 3-phosphorylated derivatives of phosphoinositides are of great interest because the enzyme responsible for their synthesis, PI-3-kinase, associates with a number of activated tyrosine-phosphorylated receptors (Cantley *et al.*, 1991) and with the ras oncogene (Rodriguez-Viciana *et al.*, 1994). PI-3-kinase has been purified to homogeneity from rat liver cytosol and the enzyme can use PI, PI-4-P and PI-4,5-P_2 as substrates (Carpenter *et al.*, 1990).

1.2.5 Regulation of phospholipid biosynthesis by CTP

CTP, a common precursor for the biosynthesis of all membrane phospholipids, plays an essential role in phospholipid biosynthesis (Figure 11). It is the direct precursor of the phospholipid biosynthetic intermediates CDP-diacylglycerol (Carter and Kennedy, 1966), CDP-choline (Kennedy and Weiss, 1956) and CDP-ethanolamine (Kennedy and Weiss,

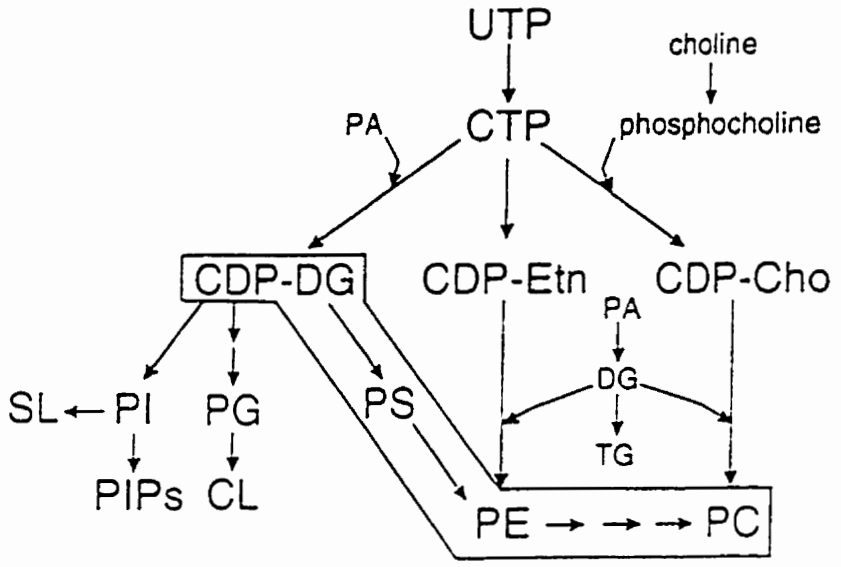


Figure 11. **The role of CTP in the biosynthesis of phospholipids.** Adapted from McDonough *et al.*, (1995) Regulation of phospholipid biosynthesis in *Saccharomyces cerevisiae* by CTP. *J. Biol. Chem.* **270**, 18774-18780.

1956). CDP-diacylglycerol is the source of the phosphatidyl moiety of PS, PE and PC synthesized by the CDP-diacylglycerol pathway (in yeast) as well as PI, PG and cardiolipin (Carman and Henry, 1989; McDonough *et al.*, 1995), while CDP-choline and CDP-ethanolamine are the sources of the hydrophilic head groups of PC and PE synthesized by the *de novo* pathways, respectively (Carman and Henry, 1989; McDonough *et al.*, 1995; Kent, 1995) (Figure 11).

CTP levels are important factors in determining the rate of phospholipid biosynthesis in mammalian cells. Choy and Vance provided the first demonstration that the elevation of PC biosynthesis in poliovirus-infected HeLa cells is due to the elevated levels of CTP (Choy *et al.*,1980). Subsequent studies have suggested that exogenous cytidine stimulates PC synthesis by increasing the level of CTP (Coviella and Wurtman, 1992; Savci and Wurtman, 1995).

In mammalian cells, CTP is synthesized through the *de novo* biosynthetic pathway from glutamine. However, cytidine or cytosine can be converted to CTP through the pyrimidine biosynthetic pathway (cytidine - cytosine - uracil - UMP - UDP - CTP) (Jund and Lacroute, 1970). CTP synthase catalyzes the transfer of an amide nitrogen from glutamine to the C-4 position of the UTP to form CTP (Long and Pardee, 1967). This enzyme plays an important role in regulating cellular CTP levels.

CTP synthase is encoded by the *URA7* (Ozier-Kalogeropoulos *et al.*, 1991) and *URA8* gene (Ozier-Kalogeropoulos *et al.*, 1994) in yeast *Saccharomyces cerevisiae*. Overexpression of *URA7* encoded CTP synthase in yeast *Saccharomyces cerevisiae* resulted in 2.4-fold elevation of the cellular concentration of CTP and a 2-fold increase in the

utilization of CDP-choline pathway for PC biosynthesis (McDonough *et al.*, 1995). This has been attributed to an increase in the availability of CTP for the PCCT reaction in the CDP-choline pathway and the inhibition of PS synthase activity by CTP in the CDP-DAG pathway (McDonough *et al.*, 1995). The apparent K_m of CTP (1.4 mM) for PCCT (Nikawa *et al.*, 1983) was 2-fold higher than the cellular concentration of CTP (0.7 mM) in control cells. This provided an explanation of why overexpression of CTP synthase caused an increase in the cellular concentration of CDP-choline and PC synthesis.

CTP synthase activity in *Saccharomyces cerevisiae* is allosterically regulated by CTP product inhibition. CTP inhibits the enzyme activity by increasing the positive cooperativity of CTP synthase for UTP (Yang *et al.*, 1994; Nadkarni *et al.*, 1995, Ostrander *et al.*, 1998). This regulation controls the cellular level of CTP in growing *Saccharomyces cerevisia* cells (Ozier-Kalogeropoulos *et al.*, 1991; Yang *et al.*, 1994; McDonough *et al.*, 1995). In mammalian cells, inhibition of CTP synthase by CTP plays an important role in the balance of pyrimidine nucleotide triphosphate pools (Ostrander *et al.*, 1998). Amino acid residue Glu^{161} in both the URA7-encoded and URA8-encoded CTP synthase was identified as being involved in the regulation of CTP synthase activity by CTP production inhibition (Ostrander *et al.*, 1998). The specific activity of the CTP synthase with a Glu^{161} →Lys (E161K) mutation was 2-fold greater when compared with the wide-type enzymes (Ostrander *et al.*, 1998). Cells carrying a E161K mutation in the CTP synthase exhibited less sensitivity to CTP product inhibition and accumulated high levels of CTP (Ostrander *et al.*, 1998). Cells bearing the mutation exhibited an increase in the synthesis of PC (1.5-fold), PE (1.3-fold), and PA (2-fold) and a decrease in the synthesis of PS (1.7-fold) due to the inhibition of PS

synthase by the elevated CTP. Moreover, cells carrying the E161K mutation exhibited 1.6-fold increase in the ratio of total neutral lipids to phospholipids when compared with control cells. All these data support that the regulation of CTP synthase activity by CTP plays an important role in the regulation of phospholipid biosynthesis.

Hatch and McClarty provided further evidence to support the notion that the cellular CTP level may be a universal switch for phospholipid biosynthesis in eukaryotic cells (Hatch and McClarty, 1996). In their study, cyclopentenylcytosine (CPEC), a cancer therapeutic drug, was used specifically to reduce the cellular pool size of CTP to less than 10% of control cells. It is well documented that upon addition to cells, CPEC is rapidly converted to CPEC-triphosphate which is a specific and potent inhibitor of CTP synthetase (Kang *et al.*, 1989). They demonstrated that the *de novo* biosynthesis of PC from [^{3}H-methyl]choline and PE from [^{3}H]ethanolamine as well as the biosynthesis of all GPLs from [U-^{14}C]glycerol or [1,3-^{3}H]glycerol were reduced to approximately 50% after preincubation of the cells with CPEC. In contrast, [U-^{14}C]glycerol or [1,3-^{3}H]glycerol accumulated in phosphatidic acid, diacylglycerol, and triacylglycerol in CPEC treated cells compared with controls suggesting a re-directing of phospholipids biosynthesis away from CTP utilizing reactions toward neutral lipid synthesis (Hatch and McClarty, 1996). Furthermore, the *de novo* biosynthesis of all phospholipids was restored to control levels by elevation of CTP levels as a consequence of the addition of cytidine to medium (Hatch and McClarty, 1996).

In addition to being used for phospholipid and RNA synthesis, CTP is converted to deoxyribocytidine triphosphate (dCTP) by ribonucleotide reductase (CTP → CDP → dCDP → dCTP). dCTP is used exclusively for DNA synthesis and repair. However, many studies

have demonstrated that dCTP can also be used as a substitute for CTP in phospholipid synthesis (Spasokukotskaja *et al.*, 1988; Sasvari-Szekely, *et al.*, 1993). It is not known how phospholipid and DNA synthesis are controlled by CTP in the cell cycle.

1.3 Regulation of PCCT

1.3.1 The structure of PCCT

PCCT is the rate limiting enzyme for PC biosynthesis in mammalian cells (Kent, 1990; Kent 1997; Tijburg *et al*, 1989) and is therefore likely to play a key role in regulating membrane PC production. cDNAs that encode PCCT have been identified and sequenced in rat (Kalmar *et.al.*, 1990), hamster (Sweitzer and Kent, 1994), mouse (Rutherford, 1993), and human (Kalmar *et.al.*, 1994). The enzyme can be divided into four distinct functional domains (Figure 12). The amino-terminal domain contains a sequence that specifies the nuclear localization of the protein between residues 2 and 28 (Wang *et al*, 1993a; Wang *et al.*, 1995). The catalytic domain from residue 72 to 233 is responsible for substrate binding and catalysis. The third domain, located between residues 256 and 288, contains three 11-residue amphipathic repeats that form α-helices following association with lipid regulators and contribute to the reversible membrane association of the enzyme (Dunne *et al.*, 1996; Arnold and Cornell, 1996; Johnson *et al.*, 1997). The fourth domain of PCCT is the carboxyl-terminal phosphorylation domain; it encompasses residues 315 and 367.

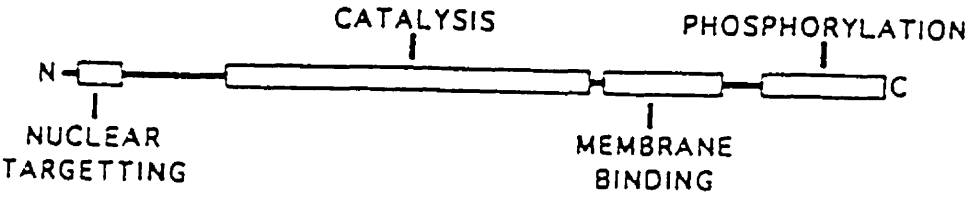


Figure 12. **Functional domains of CTP: phosphocholine cytidylyltransferase (PCCT).** PCCT can be divided into four distinct functional domains. The N-terminal nuclear targeting domain (residues 8-28); catalytic domain (residues 72-236); membrane binding domain (residues 240-290); and the C-terminal phosphorylation domain (residues 315-367). Adapted from Kent (1997) CTP:phosphocholine cytidylyltransferase. *Biochim. Biophys. Acta* **1348**, 79-90.

1.3.2 PCCT binding to membranes

PCCT is frequently characterized as having a bimodal distribution since both soluble and particulate forms of the enzyme have been reported in a number of cell types. Stimulation of PC biosynthesis is often correlated with a translocation of the enzyme from the soluble to the particulate fraction (Wright *et al.*, 1985; Wang *et al*, 1993a). The purified soluble form of the enzyme possesses low catalytic activity, but it can be fully activated *in vitro* by the addition of activating lipids, such as oleate, PG, and PI (Cornell, 1991a; Cornell, 1991b; Jamil *et al.*, 1992). While diacylglycerol alone will not activate PCCT, it can activate the enzyme when present in mixed liposomes with PC (Cornell, 1991a and1991b; Arnold *et al.*, 1997).

Treatment of cells with phospholipase C causes translocation of PCCT (Sleight and Kent, 1980; Sleight and Kent, 1983), possibly due to the production of diacylglycerol in the cell by phospholipase C. Exogenous diacylglycerol can cause the translocation of PCCT, and this translocation is independent of protein kinase C activity (Cornell and Vance, 1987; Jones and Kent, 1991), suggesting that diacylglycerol may directly recruit PCCT to the membrane. Fatty acids (Pelech *et al.*, 1984; Weinhold *et al.*, 1984; Mock *et al*, 1986; Sleight and Kent, 1983; Cornell and Vance, 1987) have been shown to increase cellular diacylglycerol levels and membrane association of PCCT. However, the ready conversion of fatty acids to diacylglycerol makes it difficult to determine the true identity of the stimulant of PCCT translocation in the cell. Addition of oleate to hepatocytes results in accumulation of diacylglycerol but not membrane-associated free fatty acid, suggesting that diacylglycerol, but not fatty acid is the true stimulant (Hatch *et al.*, 1992). However, since both lipids are

PCCT activators *in vitro*, it is likely that both may be activators *in vivo*.

PC-deficient membranes obtained by choline starvation (Yao *et al*, 1990; Weinhold *et al*., 1994) can also promote PCCT translocation to the membrane and its subsequent activation. The increase in membrane-associated PCCT in rat hepatocyte correlated with decreased PC levels without changes in the levels of diacylglycerol and fatty acids (Jamil *et al*, 1990). Therefore, it appears that altered phospholipid compositions as well as the addition of fatty acids and diacylglycerol can also cause translocation and activation of PCCT.

There is evidence to support the concept proposed by Cornell that the amphipathic α-helical domain in PCCT is responsible for lipid activation *in vitro* and membrane-binding *in vivo* (Kalmar *et al*., 1990). Rat PCCT is not activated by lipids when the amphipathic helical domain is removed by proteolysis (Craig *et al*., 1994), truncation (Yang *et al*., 1995; Wang and Kent, 1995b; Cornell *et al*., 1995), or blocked by an antibody (Wieder *et al*., 1994). Expression of PCCT that is truncated at the end of the catalytic domain results in an enzyme that cannot translocate to the membrane and is constitutively active (Wang and Kent, 1995b). These results strongly suggest that the lipid binding domain is an inhibitory domain and that the inhibition can be removed either by mutagenesis or binding to lipids or membranes. Kinetic studies have revealed that the binding of stimulatory lipids to the amphipathic helical domain of PCCT greatly enhances the catalytic activity by lowering the K_m of the enzyme for CTP to levels in the range of the cellular concentration of the nucleotide (Yang *et al*., 1995).

1.3.3 Subcellular localization of PCCT

It is commonly believed that PCCT exists in cells as an inactive soluble form and an active particulate form (membrane-associate). Activation of PC synthesis is associated with a translocation of PCCT from the soluble form to the particulate form (see section 1.3.2). However, the intracellular localization of soluble and particulate forms of PCCT has been debated. The observation that PCCT is readily liberated by digitonin permeabilization of many cell types suggests that soluble PCCT is cytoplasmic. Gradient sedimentation studies suggest that membrane associated PCCT is enriched in the endoplasmic reticulum (Wilgram and Kennedy, 1963; Tercé *et al.*, 1991) and Golgi apparatus (Higgins and Fieldsend, 1987; Vance and Vance, 1988). In light of these observations, the inactive soluble form of the enzyme was long believed to be in the cytosol while the active forms were thought to be associated with the endoplasmic reticulum (Vance, 1989).

However, the concept that PCCT is cytoplasmic has been called into question by a series experiments that indicate the enzyme is nuclear in several cell lines (Houweling *et al.*, 1996; Morand and Kent, 1989). Studies using fluorescent antibodies directed against PCCT have demonstrated that the inactive PCCT indeed appears to be located mainly in the nucleus (Houweling *et al*, 1996; Morand and Kent, 1989), while the active enzyme preferentially associates with the nuclear membrane *in vivo* in many cell lines (Chinese hamster ovary cells, Hela cells and primary hepatocytes) (Watkins and Kent, 1991; Watkins and Kent 1992; Wang *et al.*, 1993b, Utal *et al*, 1991) and in primary rat liver slices (Wang *et al*, 1995). The nuclear localization of PCCT was confirmed by enucleation studies in mouse L cells (Wang, *et al.*, 1993b). The identification of a nuclear localization domain in the amino-terminal

domain of the PCCT further supports its nuclear localization (Wang *et al.*, 1995).

The lingering discrepancies regarding the intracellular localization of PCCT may be finally resolved by Jackowski's recent report of a second human PCCT isoform (PCCTβ) (Lykidis *et al.*, 1998). The predicted molecular weight of PCCTβ is 36kDa but the protein apparently undergoes substantial post-translational modification because its demonstrated molecular weight in *vivo* is approximately 41 kDa, almost identical to that of PCCTα, the other isoform (42 kDa). PCCTβ catalyzes the same enzymatic reaction and also requires the presence of lipids for full activity. Although both PCCT isoforms share a high homology of catalytic domain (90%) and lipid binding domains (88%), it is notable that PCCTβ clearly lacks a nuclear targeting sequence and the phosphorylation domain. Indirect immunofluorescence in HeLa cells indicates that PCCTβ is cytoplasmic (Lykidis *et al.*, 1998). This finding provides an explanation for the long controversy regarding the subcellular localization of PCCT and its regulation by phosphorylation (see section 1.3.4).

1.3.4 Phosphorylation of PCCT

Although it is known that activation of PCCT, and thus, PC biosynthesis is associated with translocation of PCCT from the soluble fraction to the particulate fraction. The mechanism by which PCCT is activated remains unclear. The initial speculation that PCCT is regulated by phosphorylation was based on the observation that PC biosynthesis was inhibited by cAMP analogs (Pelech *et al.*, 1981). This speculation was supported by the finding that cAMP-dependent protein kinase could phosphorylate and inactivate PCCT *in vitro* (Sanghera and Vance, 1989). Using immunoprecipitation of ^{32}P-labeled HeLa cells,

PCCT was demonstrated to be phosphorylated (Watkins and Kent, 1990). Since phorbol esters and diacylglycerol, activators of protein kinase C, were known to stimulate PC biosynthesis, it seemed reasonable that protein kinase C might modulate PCCT activity by phosphorylation. However, subsequent studies demonstrated that neither cAMP-dependent kinase (Cornell, 1991b; Jamil *et al.*, 1992; and Watkins *et al.*, 1992) nor protein kinase C (Vance, 1990; Watkins and Kent, 1990; Watkins and Kent, 1992) phosphorylate PCCT *in vivo*.

In the search for agents that modulate PCCT phosphorylation. It was demonstrated that many of the agents promoting PCCT translocation also induce a dephosphorylation of PCCT (Wang *et al*, 1993a; Weinhold *et al.*, 1994; Watkins and Kent, 1991; Houweling *et al.*, 1994). Addition of oleate to HeLa cells caused a 10-fold stimulation of [^{3}H]choline incorporation into PC with a concomitant translocation of PCCT from the soluble to the particulate fraction. Immunoblot analysis revealed that the soluble form consisted of multiple slowly migrating bands while the particulate form was a single fast migrating band. The slowly migrating form could be converted to the fast migrating form by phosphatase treatment, suggesting that the mobility difference was due to phosphorylation (Wang *et al.*, 1993a). In addition, *in vivo* ^{32}P labeling showed that the soluble PCCT was highly phosphorylated while the particulate form was much less phosphorylated (Wang *et al.*, 1993a). Stimulation of PC synthesis in Chinese hamster ovary cells with exogenous PC-specific phospholipase C triggers the dephosphorylation and membrane association of PCCT (Walkins and Kent, 1991; Houweling *et al.*, 1994), whereas pretreatment of cells with protein phosphatase inhibitor, okadaic acid, blocks PCCT dephosphorylation and prevents

the stimulation of PC synthesis (Watkins and Kent, 1991; Hatch *et al.*, 1991). The correlation between increased PCCT phosphorylation and decreased PC synthesis *in vivo* strongly suggests that PCCT activity is physiologically regulated by reversible phosphorylation (Hatch *et al.*, 1991). Together, all these results indicate that phosphorylation and dephosphorylation may be a general mechanism for regulating PCCT activity, subcellular localization and therefore PC biosynthesis.

The identification of the carboxyl-terminal phosphorylation domain in PCCT (residues 315 to 367) further supports the concept that PCCT is regulated by phosphorylation. There are sixteen serine residues and are all phosphorylated to some extent in rat PCCT expressed in a baculovirus system (McDonald and Kent, 1994). Seven of the sixteen phosphorylation sites consist of Ser-Pro, suggesting that proline-directed kinases may be involved in the phosphorylation of PCCT. Conversion of the seven Ser-Pro sequences to Ala-Pro abolishes phosphorylation at all but one site (Wang and Kent, 1995a), indicating proline-directed kinases are likely to control PCCT phosphorylation *in vivo*. Whereas conversion of all sixteen Ser residues to Ala or Glu completely eliminates phosphorylation of PCCT, confirming that all the phosphorylation sites are limited to the C-terminal domain. In the search for kinases that phosphorylate and regulate PCCT, Wieprecht *et al* have tested the ability of three proline-directed kinases, $p34^{cdc2}$ kinase, casein kinase II, and MAP kinase, in phosphorylating rat liver PCCT *in vitro* (Wieprecht *et al.*, 1996). All three kinases were able to phosphorylate PCCT *in vitro* with $p34^{cdc2}$ producing the strongest phosphorylation. Inclusion of olomoucine, a cyclin-dependent kinase (Cdk) specific inhibitor, resulted in a concentration-dependent inhibition of phosphorylation (Wieprecht *et al.*, 1996). Taken

together, all these finding suggest that cyclin-dependent kinase may play a role in phosphorylating PCCT and therefore regulating PC synthesis in the cell cycle.

While there is a clear correlation of the extent of dephosphorylation with membrane-association and activation of PCCT, it is not clear which one of these three phenomena is causative. To test the possibility that dephosphorylation triggers the association of PCCT with membrane and subsequent activation, a number of PCCT mutants that lack the phosphorylation sites were created by site-directed mutagenesis (Wang and Kent, 1995a) and by truncation of the wild-type PCCT (Wang and Kent, 1995b). The mutant proteins were expressed in a CHO cell line. Fractionation of cells expressing the phosphorylating-deficient enzymes revealed that the level of membrane associated PCCT was higher with the mutant enzymes than with the wild-type enzyme, but a large amount of mutant PCCT was soluble. The soluble mutant forms were capable of translocating upon fatty acid stimulation. These results clearly demonstrated that dephosphorylation does not trigger membrane association and is consistent with the early report that dephosphorylation of PCCT is not required for membrane binding (Houweling *et al.*, 1994). The fact that more PCCT was associated with membranes in phosphorylation-deficient mutants suggests that dephosphorylation may stabilize the enzyme in membrane form (Wang and Kent 1995a; Wang and Kent 1995b), whereas phosphorylation attenuates PCCT activity by interfering with membrane association in cells (Yang and Jackowski, 1995).

1.4 Phospholipid catabolism

1.4.1 Introduction

As mentioned previously, membranes are an essential component of all cells since they define cell identity and subdivide the cell into specialized compartments. Phospholipids are the major building blocks of biological membranes. In addition to their structural role, phospholipids serve as an important reservoir from which cells generate intracellular and intercellular messengers, such as eicosanoids, platelet-activating factor, lysophosphatidic acid, diacyglycerol, inositol phosphates, and phosphatidic acid. These lipid messengers have been implicated in fundamental cellular responses including growth, differentiation, adhesion, migration, secretion and apoptosis (Rizzo *et al.*, 1999; Liscovitch and Cantley, 1994; Waite, 1991; Larrodera *et al.*, 1990). All pathways that lead to the generation of these lipid messengers are initiated by phospholipases.

Phospholipases are a group of enzymes that share the common property of hydrolyzing ester bonds in phospholipids. Different phospholipases hydrolyze specific ester bonds in phospholipids. Phospholipases are classified as belonging to A, B, C, or D classes based on the bond they hydrolyze (Figure 13). Some of the functions that phospholipases carry out include digesting extracellular phospholipids, generating bioactive molecules or precursors of bioactive molecules, and membrane phospholipid remodeling.

1.4.2 Phospholipase A

Phospholipase A (PLA) catalyzes the hydrolysis of two acyl groups from phospholipids and are subdivided into phospholipase A_1 (PLA_1) and phospholipase A_2

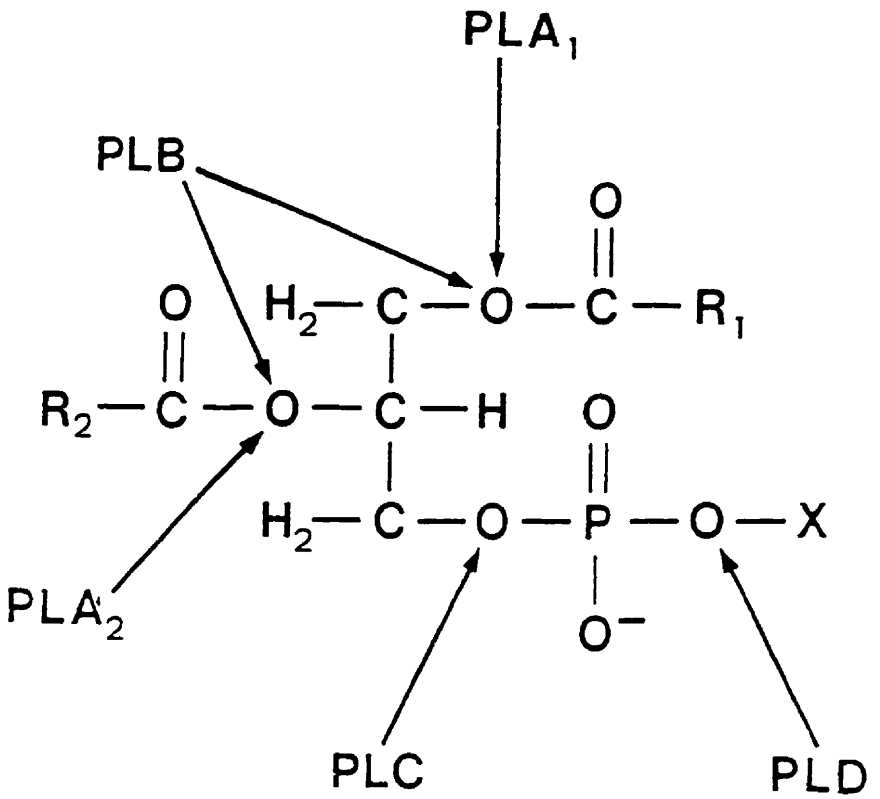


Figure 13. **Phisoholipid hydrolysis by phospholipases.** Adapted from Waite (1997) Phospholipids and phospholipases. In *Advances in Lipobiology*, **2**, 323-350.

(PLA_2) according to which acyl group is hydrolyzed. PLA_1 catalyzes the hydrolysis of acyl groups at the *sn*-1 position, while PLA_2 hydrolyzes the acyl groups at the *sn*-2 position. Some phospholipases hydrolyze both acyl groups and are termed as phospholipase B (PLB) (Waite, 1991 and 1997). In addition, lysophospholipase catalyzes the removal of one or the other acyl groups from lysophospholipids and this enzyme will be discussed in the next section. PLAs are important not only in phospholipid degradation but also in the turnover of acyl groups and in the release of fatty acids for particular purposes such as eicosanoid production (Smith *et al.*, 1991).

Of the three types of PLAs (PLA_1, PLA_2 and PLB), PLA_2 has been the best studied (Dennis, 1994). The extracellular PLA_2s are found in secretory granules, snake and bee venoms and digestive fluids of higher organisms. These enzymes have relatively low molecular weights (10-20 kDa) and are not believed to be involved in cell signaling. The intracellular PLA_2s are found in plasma membranes and other intracellular membranes as well as the cytosol. Mammalian cells contain structurally diverse forms of PLA_2s including the well characterized Groups I, II, and III small Ca^{2+}-dependent secretory PLA_2s ($sPLA_2$), the 85-kDa Group IV Ca^{2+}-dependent cytosolic PLA_2s ($cPLA_2$), and the 80-kDa Ca^{2+}-independent cytosolic PLA_2s ($iPLA_2$) (Dennis, 1994). The presence of diverse PLA_2 enzymes in mammalian cells provides multiple, differentially regulated pathways for the important process of phospholipid and fatty acid turnover (Dennis, 1994).

$cPLA_2$ is a widely distributed enzyme in all human tissues examined. It plays an essential role in mediating arachidonic acid release, eicosanoid production and signal transduction (Leslie,1997; Kramer and Sharp, 1997). $cPLA_2$ does not share homology with

other PLA_2 enzymes and it is the only well characterized PLA_2 that preferentially hydrolyzes *sn-2* arachidonic acid from PC (Clark *et al.*, 1991; Sharp *et al.*, 1991). The important role of arachidonic acid in cellular activation ensures that its levels are tightly controlled. $cPLA_2$ is therefore subject to complex regulation mechanisms. Upon activation, $cPLA_2$ is translocated from the cytosol to the nuclear envelope and endoplasmic reticulum (Schievella *et al.*, 1995; Pouliot *et al.*, 1996). $cPLA_2$ requires calcium for activity; however, unlike $sPLA_2$, calcium is needed for binding $cPLA_2$ to membrane or phospholipid vesicles rather than for catalysis. Agonist-induced phosphorylation of $cPLA_2$ on Ser residues has been observed, and the increased $cPLA_2$ activity in stimulated cells is attributed to phosphorylation on Ser^{505} by mitogen-activated protein kinase (MAPK) (Lin *et al.*, 1992; Qiu *et al.*, 1993; DeCarvalho *et al.*, 1996). Although phosphorylation on Ser^{505} is important for $cPLA_2$ activation in certain cells, it is not sufficient for full activation leading to arachidonic acid release. In macrophages, MAPK is activated by CSF-1 which in turn induces phosphorylation and an increase in the activity of $cPLA_2$. However, CSF-1 does not induce arachidonic acid release, this requires the presence of calcium-mobilizing agonists (Xu *et al.*, 1994). This suggests that an increase in intracellular calcium, necessary for $cPLA_2$ translocation, and $cPLA_2$ phosphorylation probably act together to fully activate $cPLA_2$.

Unlike $cPLA_2$, $sPLA_2$ and $iPLA_2$ do not exhibit acyl chain specificity. It is commonly believed that $iPLA_2$ is responsible for fatty acid remodeling under resting conditions (Dennis, 1994; Balsinde and Dennis, 1997). There is an ongoing deacylation/reacylation cycle of membrane phospholipids (membrane phospholipid remodeling), whereby a pre-existing phospholipid is cleaved by an intracellular PLA_2 to generate a lysophospholipid, which in

turn may be reacylated with a different fatty acid to generate a new molecular species of phospholipid.

In addition to its role in phospholipid remodeling in resting cell, $iPLA_2$ has been identified as the major phospholipase that is involved in removing excess phospholipid resulting from overexpression of PCCT (Walkey *et al.*, 1994; Baburina and Jackowski, 1999; Barbour, *et al.*, 1999). PC is the major membrane phospholipid and its synthesis is controlled by the activity of PCCT. Overexpression of PCCT induces a 20 ~ 100 fold increase in the levels of microsomal PCCT activity and a 3 ~ 5 fold increase in the rate of [^{3}H]choline and [^{3}H]glycerol incorporation into PC in COS cells (Walkey *et al.*, 1994). However, only a 17% increase in PC mass is observed. The small increase in PC mass is attributed, in part, to a dramatic increase in the rate of PC degradation which is associated with an elevation of glycerophosphocholine (GPC). Neither the diacyglycerol and phosphatidic acid mass was altered (Walkey *et al.*, 1994). This study suggests that the increased PC synthesis is compensated for by accelerating PC catabolism *via* PC → lysoPC → GPC, catalyzed by PLA and lysophospholipase. Similar results have recently been reported by Baburina and Jackowski who has used an inducible expression system to overexpress PCCT in HeLa cells (Baburina and Jackowski, 1999). Morash *et al.* have also demonstrated that PC is primarily catabolized to GPC in murine neuroblastoma cells, rat glioma cells and primary human fibroblasts (Morash *et al.*, 1988). The accumulation of GPC induced by enforced PCCT expression in Baburina and Jackowski's study can be blocked in cells treated with bromoenol lactone, a calcium-independent PLA_2 inhibitor. More recently, it was reported that the $iPLA_2$ activity as well as its protein level were up-regulated

in CHO cells that overexpressed PCCT (Barbour, *et al.*, 1999). Taken together, all these data suggest that $iPLA_2$ is the key PLA involved in PC degradation.

1.4.3 Lysophospholipase A

Lysophospholipase A (lysoPLA) are widely distributed enzymes that catalyze the hydrolysis of lysoPLs to yield a glycerophosphate derivative and a fatty acid. This enzyme has been recently extensively reviewed (Wang and Dennis, 1999). The *in vivo* levels of lysoPLs do affect cell survival and function, since the accumulation of lysoPLs can perturb the activities of many membrane-bound enzymes (Shier *et al.*, 1976; Kelly R.A., 1986), distort cell membrane integrity, and even cause cell lysis (Weltzien 1979). Several enzymes are involved in regulating lysoPL levels, including lysoPLAs, acyltransferases and transacylases. However, lysoPLA hydrolysis is considered to be the major route by which lysoPLs are removed because of their relatively high activities (Zhang and Dennis, 1988; Ross and Kish, 1994, Wang and Dennis, 1999). LysoPLA activity has been identified in many mammalian tissues and cells, including the rabbit heart (Gross and Sobel, 1983), rat and beef liver (Sugimoto *et al.*, 1996; DeJong *et al.*, 1974), murine macrophage P388D1 (Zhang and Dennis, 1988; Zhang *et al.*, 1991), WEHI 265.1 (Garsetti *et al.*, 1992a), and human HL60 cells (Garsetti *et al.*, 1992b). Many of these tissue and cells have two small lysoPLAs (lysoPLA I and lysoPLA II) that are similar in size (about 25 kDa) and display many similar properties. Both enzymes act specific on lysoPL with no significant PLA, transacylase and acyltransferase activity (Zhang and Dennis, 1988; Zhang *et al.*, 1991; Gross and Sobel, 1983; Garsetti *et al.*, 1992a; Garsetti *et al.*, 1992b). The activity of both lysoPLA

I and lysoPLA II is independent of Ca^{2+}, Mg^{2+}, and EDTA (Zhang *et al.*, 1991; Sugimoto *et al.*, 1996; Garsetti *et al.*, 1992a).

Despite the similar molecular weight and properties between lysoPLA I and lysoPLA II, these two enzymes are distinct proteins and not splice variants or the results of post-translational modification of the same gene product. In addition, lysoPLA I displays a broader substrate specificity than lysoPLA II. LysoPLA I acts on lysoPC, lysoPE, lysoPG, lysoPI, lysoPS and PAF, while lysoPLA II prefers only lysoPC and lysoPE (Sugimoto *et al.*, 1996; Garsetti *et al.*, 1992a).

1.4.4 Phospholipase C

Phospholipase C (PLC) catalyzes the hydrolysis of a phospholipid molecule to yield diacylglycerol and a phosphobase. The ten mammalian PLC isozymes identified to date are all single polypeptides and can be divided into three types, β, γ, and δ on the basis of their structure. Four PLC-β, two PLC-γ and four PLC-δ proteins are known (Rhee and Bae, 1997). The existence of multiple forms of PLC suggests that each isozyme may differ in tissue distribution, intracellular location, regulatory mechanism and cellular functions. In fact, it has been shown that there are differences in the tissue distribution of the PLC isozymes and they couple to different signaling systems. The β-type isozymes are activated by GTP binding proteins (Taylon *et al.*, 1991; Camp *et al.*, 1992; Katz *et al.*, 1992), while the γ-type isozymes are activated through receptor-tyrosine kinases (Wahl *et al.*, 1989; Meisenhelder *et al.*,1989; Kim *et al.*, 1991). The regulatory mechanism by which PLC-δ is coupled to membrane receptors remains unclear.

Most of the studies on the role of phospholipid metabolism in the control of cell proliferation have focused on PI turnover. This pathway is initiated by the hydrolysis of a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PI-4,5-P_2) by PI-phospholipase C (PI-PLC) which generate at least two second messengers (Berridge, 1987): inositol 1,4,5-trisphosphate and diacylglycerol. Inositol 1,4,5-trisphosphate participates in the control of intracellular Ca^{2+} levels (Berridge and Irvine, 1989), whereas diacylglycerol is an important activator of protein kinase C which has been shown to be a key enzyme in mitogenic signaling (Nishizuka, 1984 and 1986).

A number of reports have suggested the existence of PI-independent signal transduction pathways involving the hydrolysis of PC for generation of diacylglycerol (Fisher and Mueller, 1968; Mufson *et al.*, 1981; Guy and Murray, 1982). Evidence for this includes the observation that the production of diacylglycerol in response to some stimuli exceeds that attributable to PI-4,5-P_2 hydrolysis (Bocckino *et al.*, 1987). This production of diacylglycerol occurs in conjunction with an increase of choline and phosphocholine, suggesting that diacylglycerol is derived from PC hydrolysis either by the action of PC-PLC or *via* PLD to first yield PA which is subsequently cleaved by PA phosphohydrolase (Bocckino *et al.*, 1987). An increasing number of agonists have been identified as potent stimulants of PC-hydrolyzing PLC activity (Bocckino *et al.*, 1985; Besterman *et al.*, 1986; Irving and Exton, 1987; Slivka *et al.*, 1988). Therefore, it is now commonly believed that the agonists that hydrolyze PI also promote PC hydrolysis in their target cells (Exton, 1990). Thus, a typical response patten is a biphasic increase in diacylglycerol, with an initial, rapid, transient peak followed by a more slowly developing, but prolonged, accumulation (Exton, 1990). The first

peak is due to PI hydrolysis and is associated with an increase in inositol phosphates and cytosolic Ca^{2+}, while the second peak is due to PC hydrolysis and is associated with an increase in choline and/or phosphocholine.

1.4.5 Phospholipase D

Phospholipase D is present in bacteria, fungi, plants, and animals. PLD acting upon PE (Kiss and Anderson, 1989) or upon PI (Balsinde and Mollinedo, 1990; Huang *et.al.*, 1992) have been described. However, the best studied enzyme is the PLD specific for PC. Activated PLD catalyzes the hydrolysis of PC to generate phosphatidic acid and choline. Besides its recently discovered role as a second messenger (Kiss, 1990), phosphatidic acid can be dephosphorylated by phosphatidate phosphohydrolase to yield diacylglycerol (Kent, 1995). In the presence of primary alcohols, PLD also catalyzes a phosphatidyl transfer reaction in which an alcohol acts as nucleophilic acceptor in place of H_2O (Kent, 1995; Exton, 1999). The resulting production of phosphatidyl alcohol represents a specific assay for PLD.

Growth factor-mediated activation of PLD has been well documented and occurs in response to a broad class of mitogens, including insulin, platelet-derived growth factor, epidermal growth factor and vasopressin (Ben-Av *et al.*, 1993; Donchenko *et al.*, 1994; Yeo and Exton, 1995). Activation of PLD occurs through interaction with the small G-proteins of the ADP-ribosylation factor (Brown *et al.*, 1993; Hammond *et al.*, 1995) and Rac/Rho families (Malcolm *et al.*, 1994) as well as with protein kinase C (Singer *et al.*, 1997). The relative contribution of these factors to the activation of PLD is highly dependent on the cell

types and the signaling model examined.

PLD could exert its biological effects by several mechanisms. The first is by changing the properties of cellular membranes by altering their lipid composition. By changing the PC and phosphatidic acid content, the physical properties of the membranes could be substantially changed. Such changes could influence a number of cellular functions, including carrier-mediated transport, the properties of certain membrane-bound enzymes, receptor binding, phagocytosis, endocytosis, prostaglandin production and cell growth (Spector and Yorek, 1985). Phosphatidic acid generated by PLD activity could interact with proteins located in the membrane or cytosol and change their activity. It was recently demonstrated that PLD generated phosphatidic acid could induce Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway (Rizzo *et al.*, 1999). A third mechanism of biological action of PLD arises from the fact that PA is rapidly converted to diacylglycerol in most cells through the action of phosphatidic acid phosphohydrolase (Kent, 1995). Thus the late phase activation of PKC produced by agonists in many cells is mainly attributable to diacylglycerol derived from PLD action (Exton, 1994). A fourth function of PLD is the generation of lysoPA through the action of a specific PLA_2 on phosphatidic acid. LysoPA has been postulated to be a second messenger (VanCorven *et al.*, 1989; Moolenaar 1991; VanCorven *et al.*, 1992).

1.5 The eukaryotic cell cycle

1.5.1 Introduction

Cells reproduce by duplicating their cellular content, such as chromosomes, organelles, and biological membranes, doubling their size and then dividing in two. This cycle, the cell division cycle, is traditionally divided into four phases: G1, S, G2 and M (mitosis) (Figure 14). Cells replicate their genomic DNA during S phase and divide into two daughter cells in mitosis (Murray and Hunt, 1993). G1 and G2 phases, the two gaps between M and S phase, provide additional time for cell growth (Murray and Hunt, 1993; Norbury and Nurse, 1992). During the G_1 phase, cells monitor their environment and size to determine whether they should commit to DNA synthesis. If the conditions are not favourable for the cells to induce DNA replication, such as the lack of serum growth factors, the G1 cells can pause in their progress around the cell cycle and enter a resting state, called G0 (quiescent state), where they can remain for days, weeks, or even years before resuming proliferation. The G2 phase provides extra time for cell growth and also ensures that DNA replication is completed before cells enter mitosis.

Rapidly dividing human cells have a cell cycle that lasts about 24 hours. G1 lasts 12 hours, S phase 6 hours, G2 phase 5-6 hours and mitosis about 30 minutes to 1 hour (Norbury and Nurse, 1992). Cells in S phase can easily be recognized by incubating cells with ^{3}H-thymidine or bromo-deoxyuridine (BrdU), an artificial thymidine analog and identifying those whose nuclei have incorporated the compounds by autoradiography (^{3}H-thymidine) or by staining with anti-BrdU antibody, respectively. Alternatively, one can distinguish cells at different phases of the cell cycle by measuring their DNA content, which doubles during

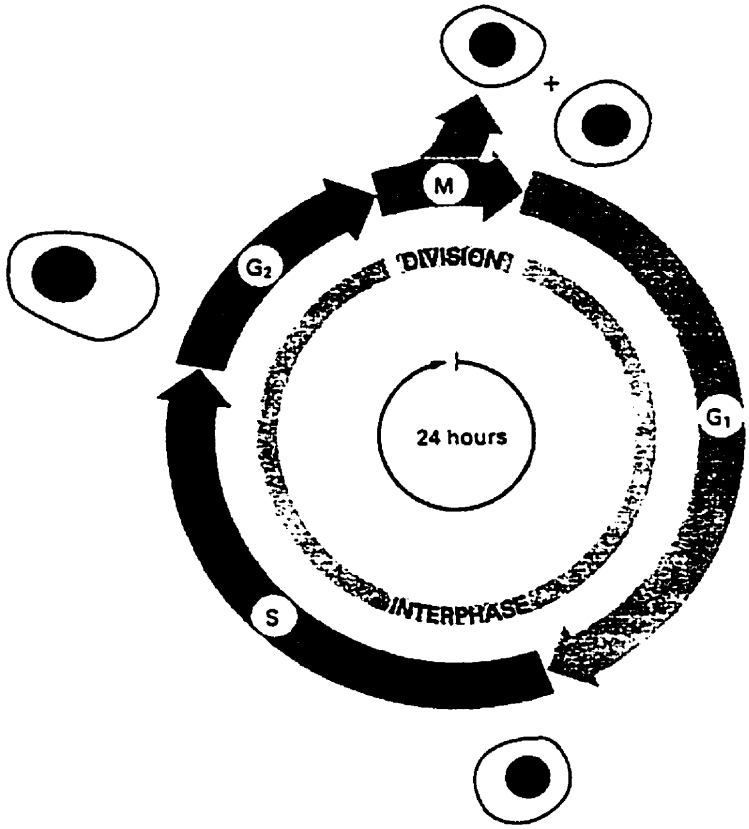


Figure 14. **The eukaryotic cell cycle.** Adapted from Alberts *et al.*, (1994d) The cell-division cycle. In *Molecular Biology of the Cell*, 3rd Ed., Garland Publishing, Inc., New York. pp. 865.

S phase. This approach is greatly facilitated by the use of a fluorescence-activated cell sorter (FACS), which allows large number of cells to be analyzed automatically. Cells in G0/G1 phase contain 2N DNA, whereas cells in G2 and M phase contain 4N DNA and S phase cells have 2N to 4N DNA (Figure 15).

1.5.2 Regulation of the eukaryotic cell cycle

The cell cycle control system is a cyclically operating system of a set of interacting proteins that induce and coordinate the essential downstream processes that ultimately result in cell division. The control system is regulated by internal and external signals that can initiate and terminate the cell cycle progression at specific checkpoints. The cell cycle is regulated at two major checkpoints, the G1 checkpoint and the G2 checkpoint. The G1 checkpoint, also termed START in yeast, is located in late G1 phase. It is responsible for determining whether cells are big enough and whether the environment is favourable for the cell to initiate DNA synthesis. The G2 checkpoint, located before the entry to mitosis, ensures that cells have completely duplicated all their genomic DNA before entering mitosis. Checkpoints respond to damage by arresting the cell cycle to provide time for repair and by inducing transcription of genes that facilitate repair (Elledge, 1996). Checkpoint loss results in genomic instability and has been implicated in the evolution of normal cells to cancer cells (Sherr, 1996).

Normal cell cycle progression requires both expression of various proteins and the regulation of preexisting proteins by phosphorylation/dephosphorylation at specific stages of the cell cycle. For example, the synthesis of dihydrofolate reductase, thymidine kinase,

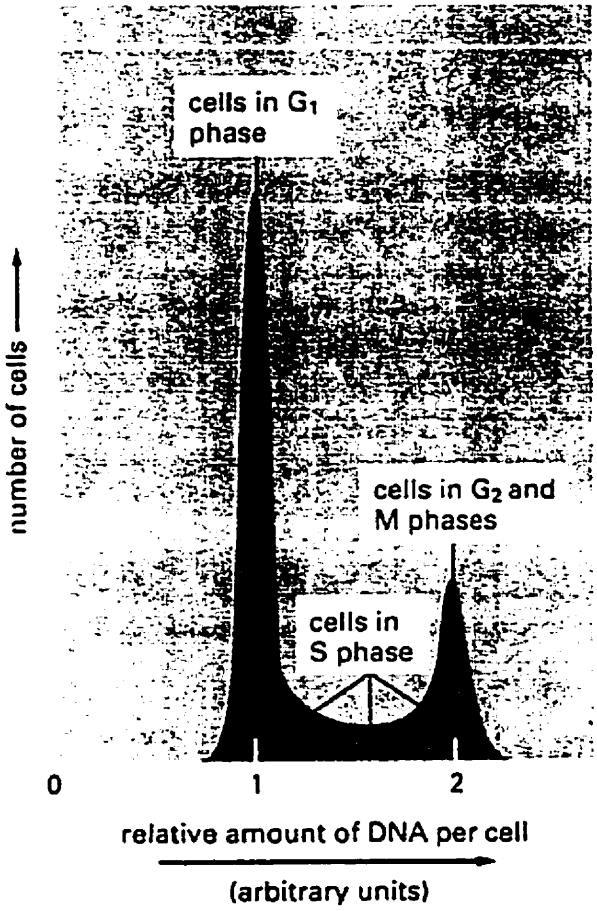


Figure 15. **Cell cycle analysis with fluorescence-activated cell sorter**

DNA polymerase δ subunit PCNA (proliferating cell nuclear antigen), and DNA polymerase α is induced in late G1 and during S phase when these enzymes are required for DNA synthesis (Alberts *et al.*, 1994d; Sherr, 1996). Phosphorylation of the retinoblastoma protein (Rb), a nuclear phosphoprotein that controls the G1 checkpoint, must occur in late G1 phase while phosphorylation of chromosomal scaffold proteins, such as histone H1, and nuclear lamins, on the other hand, have to take place when the cell enters mitosis. The processes are triggered by the complexes of cyclins (A-, B-, D- and E type) as well as their cyclin dependent kinase (Cdk) partners: Cdk1 (also known as Cdc2 and $p34^{cdc2}$), Cdk2, Cdk4 and Cdk6 (Norbury and Nurse, 1992; Stillman, 1996; Edgar and Lehner, 1996). Different cyclins associate with and transiently activate their Cdks at different stages of the cell cycle to perform various functions (Figure 16).

Cyclins are so named because their cellular levels vary during the cell cycle due to cyclical synthesis and degradation (Alberts *et al.*, 1994d; Pine, 1995; Sherr 1996). The Cdks are regulators of the cell cycle whose activities rely on the association with cyclins. Activated Cdks induce downstream processes by phosphorylating selected proteins on Ser and Thr residues. Although most known Cdks are involved in cell cycle control, the list of Cdks involved in other processes is growing (Kaffman *et al.*, 1994; Schneider *et al.*, 1994; Tsai *et al.*, 1994).

1.5.3 Regulation of Cdk activity

The periodic activation of the Cdk/cyclin complexes in the cell cycle is achieved by regulation at many levels. The complexity is regulated to ensure that the events of the cell

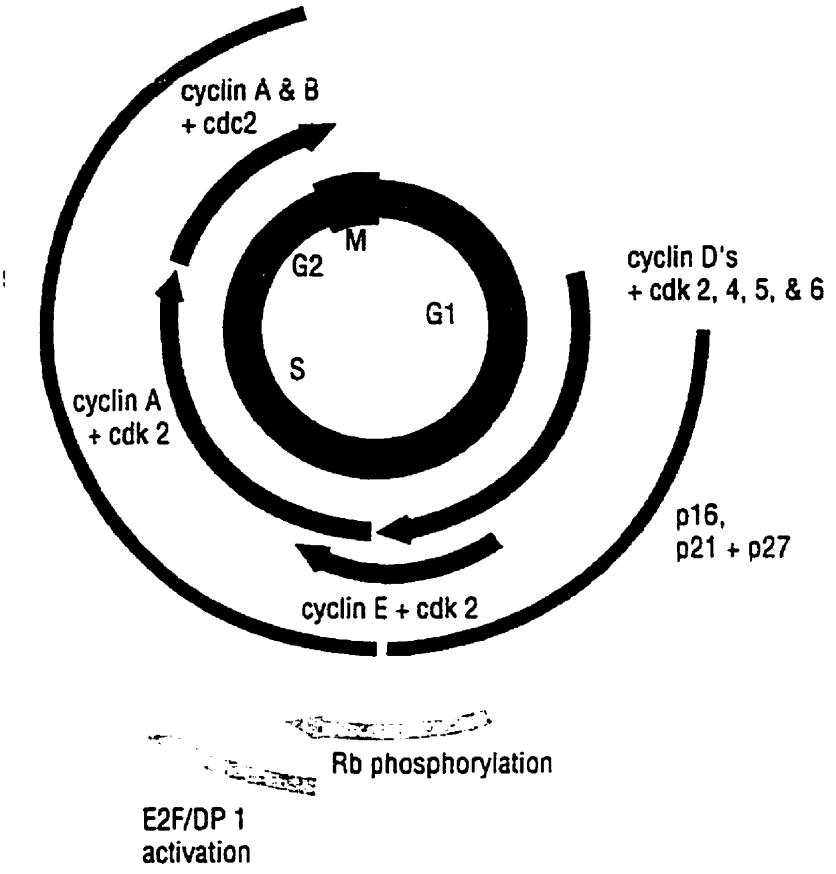


Figure 16. **Regulation of eukaryotic cell cycle by cyclin-dependent kinases**

cycle occur in the proper sequence and the appropriate time. In particular, the integrity of the DNA must be maintained by the complete replication of the entire genomic DNA before cell division in mitosis. Cdks activity are strictly controlled by several complex mechanisms (Figure 17). While cellular Cdk levels tend to remain in constant excess through the cell cycle, the concentration of the activating cyclin subunits are modulated both transcriptionally and through periodic, ubiquitin-dependent proteolysis (Sherr, 1996; King *et al.*, 1996). Cdk activation requires cyclin binding and the Cdk-cyclin complexes are subjected to both activating and inhibiting phosphorylation (Morgan, 1995; Pine, 1995). Cdk-cyclin complexes can be activated by phosphorylation of conserved Thr residues, Thr160 and Thr161, by Cdk-activating kinase (CAK) (Fisher and Morgan, 1994). CAK, like its substrate, is a Cdk-cyclin complex whose catalytic subunit is a highly conserved Cdk-related protein kinase termed MO15 (Poon *et al.*, 1994; Fisher and Morgan,1994; Solomon *et al.*, 1993), the second subunit of CAK is a new cyclin, termed cyclin H (Fisher and Morgan, 1994). MO15 has therefore been renamed Cdk7 (Fisher and Morgan,1994; Solomon *et al.*, 1993). The active Cdk-cyclin complex can be inhibited by phosphorylation of a conserved threonine-tyrosine pair, Thr14 and Tyr15. The kinase primarily responsible for Thr14 and Tyr15 phosphorylation is *weel* kinase which itself is regulated by phosphorylation (Atherton-Fessler *et al.*, 1993). Thus, after achieving the necessary prerequisites for activation, cyclin binding and CAK phosphorylation, Cdks can still be held in an inactive state by *weel* phosphorylation at Thr14 and Tyr15. Activation of the Cdk/cyclin complexes can then be accomplished by dephosphorylation of Thr14 and Tyr15 residues by Cdc25 (Dunphy, 1994). Furthermore, Cdks activity can also be inhibited by binding to Cdk inhibitor subunits, such

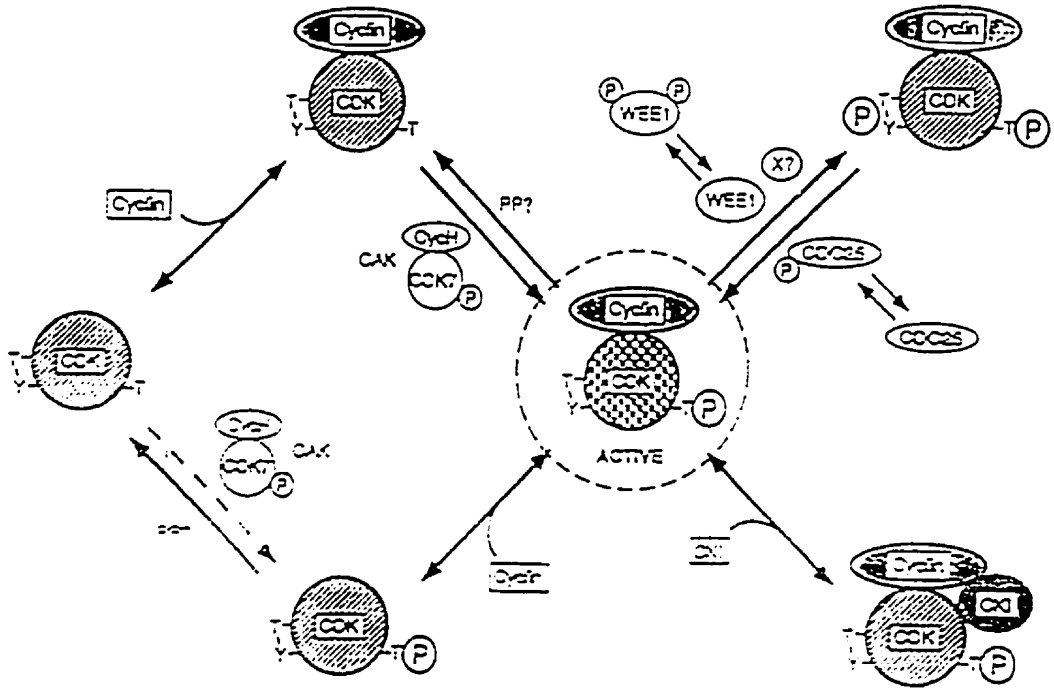


Figure 17. **Principles of cyclin-dependent kinase regulation.** Adapted from Morgan (1995)

as p21 (Xiong *et al.*,1993), p27(Toyoshima and Hunter, 1994), $p16^{INK4}$(Serrano *et al.*, 1994) and $p15^{INK4B}$(Hannon and Beach, 1994).

1.5.4 Regulation of cell progression through the G1 checkpoint

The cyclic association, activation and disassociation of cyclin-Cdk complexes are the pivotal events that drive the cell cycle. D-type cyclins in complex with Cdk4 and Cdk6 regulate cell progression through the G_1 phase of the cell cycle. There are three types of D cyclin (D1, D2, and D3), and they are cell type specific, with most cells expressing D3 and either D1 or D2 (Sherr, 1993). Removal of colony-stimulating factor 1 from macrophages resulted in immediate cessation of D1 cyclin synthesis (Matsushime *et al.*, 1991). This suggests that D-type cyclins may act as growth factor sensors. Cdk4 and Cdk6 induce cell cycle progression through G1 phase *via* phosphorylation and inactivation of Rb protein (Figure 18).

Rb is the most important inhibitor of the cell cycle progression that controls the G1 checkpoint. Rb is hypophosphorylated during G0 and early G1, and is phosphorylated by the complex of cyclin D/Cdk4 and cyclin D/Cdk6 at late G1 phase. It remains in its hyperphosphorylated form for the rest of the cell cycle until the cell emerges from mitosis (DeCaprio, 1989; DeCaprio, 1992). Rb inhibits cell cycle progression through binding to E2F and its dimerization partner (DP). E2F is a transcriptional activator for many genes that are required for cell cycle progression. Throughout G0 and early G1, E2F/DP is tightly complexed with hypophosphorylated Rb, and this complex acts as a transcriptional repressor. In late G1, both Rb and E2F become phosphorylated by the complex of cyclin D with Cdk4

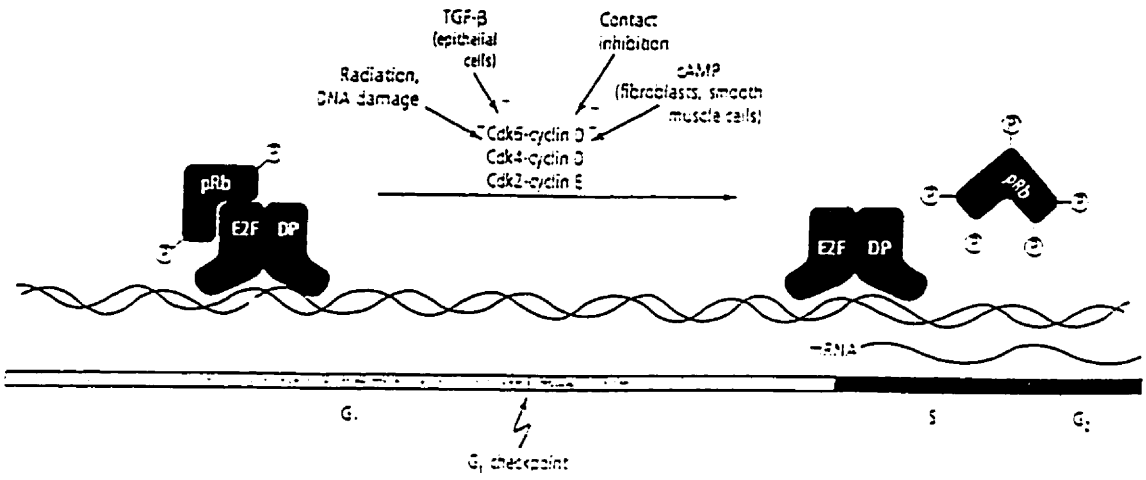


Figure 18. **Function of retinoblastoma protein (Rb) in the control of the G1 checkpoint.** Phosphorylation of Rb by CDKs releases the transcription factor E2F/DP from Rb/E2F/DP complex to initiate the transcription of genes for cell cycle progression. Growth-inhibitory stimuli prevent Rb phosphorylation by inhibiting CDKs.

and Cdk6. Phosphorylation of Rb release E2F/DP which become a transcriptional activator and induce the expression of many cell cycle dependent genes. Some products that are induced including dihydrofolate reductase, thymidine kinase, DNA polymerase δ subunit PCNA, DNA polymerase α, Cyclin E and A and even E2F itself (Alberts *et al.*, 1994d; Sherr, 1996).

In summary, mitogenic stimulation induces the synthesis of cyclin D. The cyclin D/Cdk complexes then start phosphorylating Rb; Rb phosphorylation in turn releases and activates the transcription factor E2F which then induce the expression of the E2F-regulated genes, including those for cyclin E, cyclin A and E2F itself. The complexes of Cdk2 with cyclin E and A cause further Rb phosphorylation and thereby further activates the E2F-regulated genes. Among all the events described above, only the initial event, which is the induction of cyclin D, requires mitogenic stimulation. The remaining steps are not dependent on growth factor stimulation.

1.5.5 Regulation of cell progression through S phase

The cyclin E/Cdk2 and cyclin A/Cdk2 complexes are the next Cdks to appear in the mammalian cell cycle after the D-types (Dulic, *et al.*, 1992) at late G1 phase. Both cyclin E and cyclin A are induced by cyclin D/Cdk4 and Cdk6 through Rb and E2F in late G1 as discussed in the previous section (Section 1.4.3). In addition to their role in phosphorylation of Rb, cyclin E/Cdk2 is thought to play an important role in the initiation of DNA replication while cyclin A/Cdk2 regulates cell progression through S phase (Heichman and Roberts, 1994). The physiologic substrates for Cdk2 have not yet been identified, but the cyclin

E/Cdk2 and cyclin A/Cdk2 have been shown to be associated with E2F and two Rb-related proteins, p107 (Lees *et al.*, 1992) and p130 (Hannon *et al.*, 1993), this suggests that Cdk2 might indirectly function to regulate gene expression temporally during late G1 and S phases by phosphorylating E2F itself (Lees *et al.*, 1992; Nevins, 1992). Furthermore, overexpression of cyclin E has been shown to moderately accelerate cell entry into S phase (Ohtsubo and Roberts, 1993; Resnitzky, *et al.*, 1994; Wimmel, *et al.*, 1994).

1.5.6 Regulation of cell progression through mitosis

In mitosis, nuclei undergo dramatic changes. First, the nuclear envelope breaks down, the contents of the nucleus condense into visible chromosomes, and the cellular microtubules reorganize to form the mitotic spindle that will eventually separate the chromosomes. Finally, the cell divides into two daughter cells.

Cyclin A and B in association with Cdc2 are believed to play an important role in regulating cell progression through mitosis (Dunphy, 1994; Sherr, 1994; King *et al.*, 1994). The cyclinB/Cdc2 complexes accumulate in an inactive state during S and G2 phases. The kinase is kept inactive by the inhibitory phosphorylation of Cdc2 on Tyr-14 and Tyr-15 by *Weel*/*Mik*1-related protein kinases. At the end of the G2 phase, Cdc25 phosphatase is activated by phosphorylation. Activation of Cdc25 in turn dephosphorylates Tyr-14 and Tyr-15 and thus activates Cdc2 in mitosis.

Histone H1 was the first *in vitro* substrate found for cyclin B/Cdc2 and it has been used as the standard substrate for assaying Cdc2 activity. Histone H1 is phosphorylated on specific sites at mitosis (Chambers and Langan, 1990) and it has been proposed that

phosphorylation of histone H1 may be involved in chromatin condensation (Chadee, *et al.*, 1995; Talasz, *et al.*, 1996).

Cdc25 phosphatase and protein phosphatase 1 (PP1) are two other substrates for cyclin B/Cdc2 that are potentially involved in regulating the activity of cyclin B/Cdc2 kinase itself. As mentioned above, activation of Cdc25 phosphatase by phosphorylation in late G2 leads to the dephosphorylation of Tyr-14 and Tyr-15 and activation of Cdc2 in mitosis. Activated cyclin B/Cdc2 in turn further phosphorylates Cdc25 phosphatase and thus, forms a positive feedback loop to ensure that the entry to mitosis is rapid and irreversible (Hoffmann *et al.*, 1993). Cdc25 can be dephosphorylated and inactivated by protein phosphatase1 (PP1) which has been proposed as an antagonist for Cdc25 during interphase (Izumi *et al.*, 1992). PP1 activity is lower in mitosis and this correlates with its phosphorylation, potentially by cyclin B/Cdc2 (Walker *et al.*, 1992). Protein phosphatase 2A (PP2A) has also been shown to dephosphorylate and inactivate Cdc25, suggesting that PP2A could also be a Cdc25 antagonist (Izumi *et al.*, 1992; Clarke *et al.*, 1993).

It is clear that cyclin B/Cdc2 kinase is also involved in re-organizing the architecture of the cell at mitosis. Activation of cyclin B/Cdc2 causes dramatic changes in the behaviour of the nuclear lamina, the actin microfilaments and the microtubule network. The nuclear lamina is made up of a polymer of lamin subunits that are hyperphosphorylated at mitosis, and this phosphorylation is responsible for their disassembly (Peter *et al.*, 1990a; Newport and Spann, 1987). Cyclin B/Cdc2 is a lamin kinase and is directly responsible for nuclear lamina disassembly (Peter *et al.*, 1990b). Cyclin B/Cdc2 is also involved in the re-organization of microfilaments through the phosphorylation of caldesmon (Yamashiro *et al.*,

1990; Yamashiro *et al.*, 1991; Hosoya *et al.*, 1993). It has also been implicated in the regulation of actomyosin filaments through phosphorylation of the myosin in the contractile ring which divides the cell into two (Satterwhite *et al.*, 1992). Both cyclin A/Cdc2 and cyclin B/Cdc2 kinases are believed to be involved in reorganizing the microtubule network at mitosis (Verde *et al.*, 1992).

1.5.7 Growth arrest/apoptosis in DNA damaged cells

External signals control the cell cycle progression by modulating Cdk activities which in turn regulate the Rb activity by phosphorylation (section 1.4.4.). Through its interaction with transcription factor E2F/DP, Rb thus acts as the guardian of the cell cycle. However, most growth-inhibiting external stimuli, including contact inhibition, growth inhibiting hormones, radiation or other DNA-damaging agents, do not act directly on Cdks but rather induce the synthesis or promote the activity of various Cdk inhibitors that prevent the action of cyclin/Cdk complexes on Rb (Figure 19).

p53, a tumor suppressor (Yin *et al.*, 1992), is encoded by a gene that is found to be very frequently mutated in human cancers (Sherr 1996). p53 is present in low concentrations at all times, however, DNA damage causes a sharp increase in the nuclear p53 levels by a poorly understood mechanism (Sherr 1996). p53 acts as a transcription factor that drives the expression of genes for growth arrest, DNA repair and apoptosis. The mechanism of p53-induced apoptosis is poorly understood, but the p53-dependent cell cycle inhibition is known to depend on the Cdk inhibitor p21, a nuclear protein, that inhibits various cyclin/Cdk kinases, including those that phosphorylate Rb (Xiong *et al.*,1993). p53 has antimutagenic

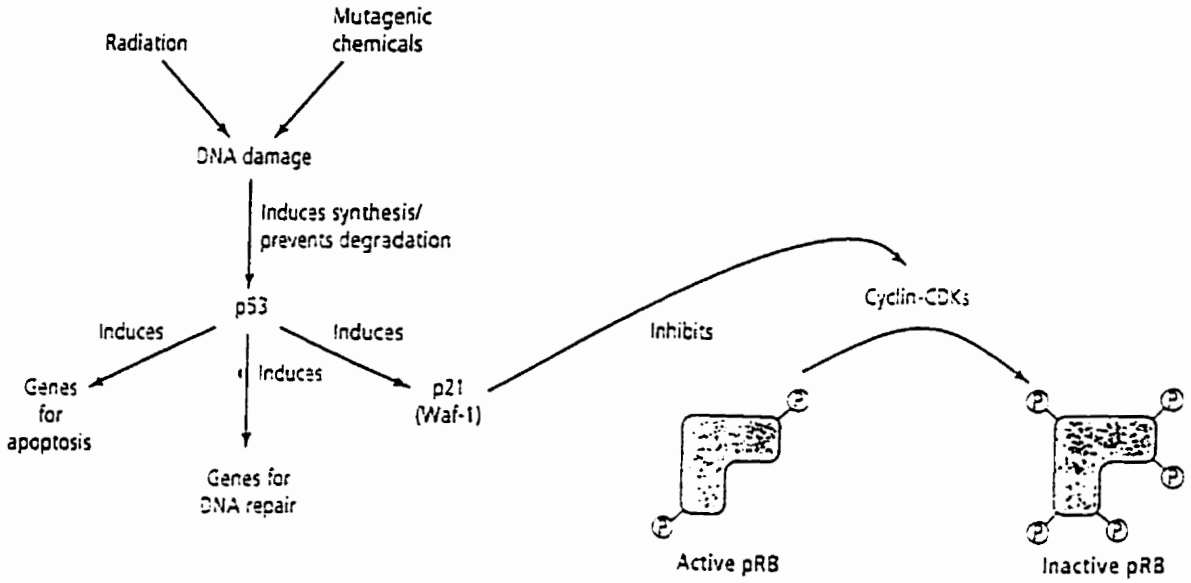


Figure 19. **The role of p53 protein in the response to DNA damage.**

properties because it delays DNA replication until DNA damage has been repaired. By inducing apoptosis, p53 prevents the survival of genetically defective cells. Therefore, p53 has been called the "guardian of the genome".

1.5.8 Cell synchronization

Because exponentially growing cells are randomly distributed at different stages of the cell cycle, they are not a suitable model to study cell cycle dependent events. To study the coordination and regulation of phospholipid production in the cell cycle, it is desirable to obtain synchronized populations of cells at different stages/phases of the cell cycle.

Many methods have been developed to synchronize cells at specific stages of the cell cycle. Serum starvation is an effective method to synchronize cells at G0/G1 (Northwood *et al.*, 1999). While starvation in methionine-free media (DeCaprio *et al.*, 1989) or incubation of cells in the presence of mimosine (Lalande *et al.*,1990), hydroxyurea (Adams and Lindsay, 1967), aphidicolin (Hubermann *et al.*,1981), or excess thymidine (Bootsma *et al.*,1964) have been widely used to synchronize cells in the G1 to S phase interval. Hydroxyurea acts on ribonucleotide reductase (Reichard and Ehrenberg, 1983), which in turn leads to cell cycle arrest at S phase by limiting the amount of ribonucleotides available for DNA synthesis (Skoog and Nordenskjöld, 1971). Aphidicolin prevents the binding of deoxyribonucleotide triphosphate to DNA polymerase α (Hubermann *et al.*,1981). Both agents efficiently arrest the S phase cells while cells at G2/M and early G1 phase which are not affected, progress normally and accumulate at the G1/S boundary. Nocodazole blocks cells at the metaphase of mitosis by destabilizing the microtubule structure and is commonly

used to arrest cells in mitosis (Zieve *et al.*,1980). Generally, these agents are very useful for cell synchronization since they arrest cells at a single specific point of the cell cycle and removal of the agent allows the cells to continue their progression in synchrony along the cell cycle. However, it should be noted that the addition of chemical agents to the cell culture may perturb biochemical processes other than the intended cell cycle event and exposure of cells to these agents for lengthy periods or at a high concentration, can lead to cell death and irreversible cell damage.

In addition to serum starvation and chemical agents used for cell synchronization, centrifugal elutriation has been used to separate cells into fractions enriched in G1, S, and G2M phase cells (Krek and Decaprio, 1995). For mammalian cells in culture, the early G1 cells are the smallest whereas cells in late G2/M are the largest. Centrifugal elutriation can separate a large number of cells into populations of uniform size. This method is more useful for suspension cells since these cells tend not to adhere to each other and they have a rounded, uniform shape.

1.6 Phospholipids and the cell cycle

1.6.1 Introduction

Cells reproduce by duplicating their cellular components, such as chromosomes, organelles and biological membrane, doubling their size and then dividing in two. Through this cycle, cells double their membrane components so that the daughter cells formed have the same membrane composition as the parent. Since GPLs are major components of the cell membrane, the increase in the membrane content will require corresponding changes in the

GPLs content. In addition, phospholipid biosynthesis is also required to replace their degradation by phospholipases during the cell cycle (Northwood *et al.*, 1999). Therefore, considerable production of GPL in the cell cycle would be expected. Relatively little attention has been directed toward defining how membrane GPL production is coordinated and regulated within the cell cycle. It was not known whether synthesis of different membrane GPL classes occurs concomitantly and continuously or in a stepwise manner in the cell cycle to maintain a constant membrane GPL composition (Jackowski, 1996). It was also not known whether synthesis of specific GPL classes occurs at different phases of the cell cycle. The biochemical mechanisms that regulate GPL production in the cell cycle are largely unknown (Jackowski, 1996). The importance of the coordinated regulation of GPL metabolism with the cell cycle is obvious, since discordant regulation of GPL accumulation by only a few percent per cell cycle would rapidly result in cells with either a large excess or deficit of membrane surface leading to abnormalities of cell size and/or intracellular lipid accumulation (Jackowski, 1996). If specific GPL classes are synthesized at different phases of the cell cycle, membrane GPL composition will vary at different stages of the cell cycle. Modification of cellular membrane GPLs composition will alter membrane fluidity (Clamp, *et al.*, 1997) and affect a number of cellular functions, including carrier-mediated transport and the properties of certain membrane bound enzymes (Spector and Yorek, 1985). Thus, stringent control mechanisms to keep the GPL content and composition in tune with the cell cycle may be expected.

1.6.2 Role of lipid-derived second messengers in the cell cycle

The research on regulation of cell cycle progression in eukaryotes has focussed mainly on the roles of CDKs in controlling specific check-points in the cell cycle. One potential role for phospholipids in cell proliferation is related to the generation of second messengers, such as diacylglycerol, phosphatidic acid and arachidonic acid, through the activation of PLC, PLD, and PLA upon growth factor stimulation. Using cytochemical methods to detect phospholipids inside the nucleus of intact cells, it was demonstrated that a significant reduction in nuclear phospholipids is associated with DNA replication (Maraldi *et al.*, 1993). The decrease in nuclear phospholipids could be due to increased phospholipid hydrolysis, whose products may in turn activate some of the enzymes involved in the control of DNA replication. In another study, York and Majerus measured the levels of nuclear PIs during the cell cycle of HeLa cells. They showed that nuclear PIs decrease by over 50% during S phase and returned to the original level at G2/M (York and Majerus, 1994). Separation of individual inositol-containing nuclear lipids showed that the content of PI decreased by 50% while the levels of PI-4-P and PI-4,5-P_2 decreased by 66%. On the other hand, the total cellular PIs remained constant throughout this period (York and Majerus, 1994). This finding further suggests that there is a specific nuclear PI turnover that is activated during DNA synthesis. While the phospholipases that are responsible for nuclear inositol lipids turnover during DNA synthesis are unknown, the recently discovered PLC δ4 (Liu *et al.*, 1996) may be a candidate. PLC δ4 is present in the nucleus and it is induced at the transition from G1 to S-phase of the cell cycle and almost disappears when cells exit mitosis (Liu *et al.*, 1996). On the other hand, the content of PLC β1, PLC γ1, and PLC δ1

do not change significantly during the cell cycle (Liu *et al.*, 1996). Some experiments have demonstrated an important role for phospholipid hydrolysis during DNA synthesis. Microinjection of two PI-PLC isozymes into quiescent NIH 3T3 cells overcame the cellular G0 block and resulted in a time- and dose-dependent induction of DNA synthesis as demonstrated by [^{3}H]thymidine incorporation into nuclear DNA (Smith *et al.*, 1989). PC-PLC mediated PC hydrolysis has also been demonstrated to be an important step in platelet-derived growth factor (PDGF) stimulated DNA synthesis in quiescent Swiss 3T3 cells by a mechanism that is independent of PKC (Larrodera *et al.*, 1990). In addition, several lipids have been shown to be potent mitogens. These include lysophosphatidic acid (VanCorven *et al.*, 1989; Moolenaar 1991; VanCorven *et al.*, 1992), phosphatidic acid (VanCorven *et al.*, 1992), and the sphingomyelin-derived compounds sphingosine (Zhang *et al.*, 1990), sphingosine-1-phosphate (Zhang *et al.*, 1991; Desai *et al.*, 1992), sphingosylphosphocholine (Desai *et al.*, 1991). Together, all these results indicate that catabolism of phospholipids could participate in regulating the cell cycle and cell proliferation.

1.6.3. Phospholipid accumulation in the cell cycle

Biosynthesis of phospholipids is an important element of the cell cycle because cells must double their membrane phospholipid content during the cell cycle. Normal cell cycle progression requires lipid which must be provided exogenously or synthesized *via* endogenous pathways. Choline, the precursor for PC biosynthesis, is absolutely required for growth of cultured cells (Glaser *et al.*, 1974) and is an essential nutrient for mammals (Zeisel *et al.*, 1991). Choline deficiency is an effective and selective method to impair PC

biosynthesis without affecting other metabolic pathways in cells (Yao *et al.*, 1990). Incubation of WI38 fibroblasts or L6 myoblasts in choline-free medium supplemented with delipidated serum resulted in cell growth arrest in the G1 phase of the cell cycle (Cornell *et al.*, 1977; Cornell and Horwitz, 1980), but 30% of cells were still able to cycle, presumably, due to residual serum lipid and/or synthesis of PC through the minor pathways (Cornell *et al.*, 1977). These observation provided the first evidence that lipids were required for cell cycle progression through G1 phase.

Tercé *et al* reinvestigated the possible requirement of PC for normal progression of cells through the cell cycle and showed that choline deficient C3H/10T1/2 fibroblasts are synchronized in late G1 phase (Tercé *et al.*, 1994). In addition, incubation of the cells in choline deficient medium causes a dramatic decrease in PC content which is correlated with a decrease in the number of cells progressing through the S phase, while the levels of PE and sphingomyelin were not affected. Normal cell replication could be restored by supplying choline or lysophosphatidylcholine which is rapidly acylated to form PC, but not other phospholipid (Tercé *et al.*, 1994). While these studies link the availability of PC or choline for the normal progression of cells along the cell cycle, the mechanism responsible for growth inhibition has not been established. How membrane GPL production is coordinately regulated in the cell cycle remains unknown. In cultured eukaryotic cells, growth factors stimulate an increase in the rate of PC biosynthesis in the early G1 phase of the cell cycle, however, the enhanced radiolabeling of PC appears to reflect mitogen-stimulated PC turnover rather than net membrane GPL accumulation (Tessner *et al.*, 1991). Steady-state [^{3}H]glycerol labelling of quiescent fibroblasts stimulated with platelet-derived growth factor

(PDGF) (Habenicht *et al.*, 1985) and total lipid phosphorus measurements in rat thymocytes treated with concanavalin A plus interleukin-2 (Gross *et al.*, 1988) revealed that greater increases in GPL mass occurred between 12 and 24 h following mitogenic stimulation than during the first 12 h. These results suggest that the net GPL accumulation may occur sometime after cells exit G1.

To investigate the possible coordination of membrane GPL accumulation with the cell cycle, BAC1.2F5 mouse macrophages were continuously labelled with [^{32}P]orthophosphate during growth in culture for two days. The cells were synchronized by deprivation of colony-stimulating factor 1 (CSF-1) from the medium. Subsequently CSF-1 was added to initiate cell cycle progression and synchronized cells at different stages of the cell cycle were harvested at various times after CSF-1 addition. Measurement of the total amount of [^{32}P]GPL revealed that there was a modest rise in the percent increase in [^{32}P]GPL during the G1 phase followed by a significant elevation in S phase (Jackowski, 1994), indicating that net phospholipid accumulation occurred in S phase. Inclusion of dibutyryl-cAMP (Rock *et al.*, 1992) or aphidicolin following CSF-1 stimulation to arrest the synchronized cells in mid-G1 or to inhibit DNA replication did not stop the increase in [^{32}P] incorporation into GPL suggesting that membrane phospholipid accumulation did not depend on the commitment of the cells to exit G1 nor the subsequent DNA synthesis (Jackowski, 1994). Further studies demonstrated that the elevation of [^{32}P]GPL in S phase resulted from an interaction between cell cycle-dependent oscillations in the rates of PC biosynthesis and degradation (Jackowski, 1994). In fact, both the biosynthesis and catabolism were occurring together in G1, but the degradation pathways were inactivated during S phase leading to the

net GPL accumulation in S phase of the cell cycle. This study, however, did not address the accumulation of any other classes of GPL in the cell cycle. It was also not established whether the membrane GPL composition remained constant or varied in the cell cycle. In addition, all radiolabelling experiments are subject to difficulties in interpretation unless equilibrium labelling can be unequivocally demonstrated, but no evidence was presented to demonstrate that BAC1.2F5 cells were labelled to equilibrium.

An alternative approach to detect changes of membrane GPL content in the cell cycle is to measure directly the mass of GPL in different stages of the cell cycle and express the value relative to the cell number. However, as far as we know, no one has actually measured changes of GPL mass in the eukaryotic cell cycle. In addition, in order to avoid the possible interference of growth factors on lipid metabolism, one should avoid using growth factor starvation to synchronize cells. Using newly developed methods to obtain synchronized cells at different stages of the cell cycle, we have re-investigated the coordination and regulation of phospholipid accumulation in the cell cycle of MCF-7 cells.

1.7 Objectives

Cells reproduce by duplicating all their cellular components, doubling their size and dividing in two. Since GPLs are major components of cellular membranes, cells must double their GPL mass so that the daughter cells formed have a same membrane composition as the parent. However, the mode of phospholipid accumulation and its regulation within the cell cycle remains largely unknown. The goal of this study is to investigate how membrane phospholipid accumulation in MCF-7 cells is coordinated with the cell cycle and how the accumulation is regulated in the cell cycle.

The specific objectives are to investigate:

[1] The coordination of membrane phospholipid accumulation with the cell cycle.

[2] The contribution of PC and PE synthesis to their accumulation in the cell cycle.

[3] The regulation of PC synthesis by PCCT in the cell cycle.

[4] The role of CTP in regulating phospholipid synthesis in the cell cycle.

[5] The contribution of catabolism to the accumulation of PC in the cell cycle.

2 MATERIALS AND EXPERIMENTAL METHODS

2.1 Materials

2.1.1 Cell lines, media and other tissue culture materials

MCF-7 (human breast adenocarcinoma) cells were grown from frozen stocks originally obtained from **American Type Culture Collection.**

Dulbecco's modified Eagle medium (DMEM) was prepared from powder purchased from **Gibco BRL.**

Fetal bovine serum (FBS) was the product of **Hyaclone.**

Bovine serum albumin (BSA) (A-9418) was purchased from **Sigma Chemical Co.**

All tissue culture ware were purchased from **Baxter Canlab Diagnostics Incorporation.**

2.1.2 Chemicals and radiochemicals

Nocodazole (T101) was purchased from **BIOMOL Research Laboratories, Inc.**

Hydroxyurea (H-8627), propidium iodide (P-4170), RNase A (500u/mg), malachite green base(M-8635), nonidet P-40 (NP-40, N-6507), choline chloride (C-7017), phosphocholine (P-0378), ethanolamine hydochloride (E-9508), phosphoethanolamine (P-0503), CDP-ethanolamine (C-0456), tween 20 (P-1379), aminoethylbenzenesulfonyl fluoride (A-8456), aprotinin (A-1153), benzamidine (B-6506), leupeptin (L-2023), were purchased from **Sigma Chemical. Co.**

CDP-choline, all phospholipids, and neutral lipids were obtained from **Doosan Serdary Research Laboratories.**

Ammonium molybdate, 70% perchloric acid, and HCl were from **Fisher Scientific Company.**

[methyl-^{3}H]Choline chloride (TRK593), [methyl-^{14}C]choline chloride (CFA424), [methyl-^{14}C]phosphocholine (CFA525), CDP-[methyl-^{14}C]choline (CFA 528), [^{14}C]ethanolamine (CFA329), [^{3}H]cytidine-5'-triphosphates (TRK339), and lysoPC[methyl-^{3}H] (ART677) were purchased from **Amersham International.**

[^{14}C]CDP-ethanolamine (#17150) was the product of **ICN Pharmaceuticals.**

LysoPC[1-palmitoyl-^{14}C](NEC683) was obtained from **American Radiolabeled Chemical Inc.**

2.1.3 Antibodies

PCCT antibody was a generous gift from Dr. Claudia Kent (Department of Biological Chemistry, University of Michigan). It was generated in rabbits using protein PCCT236 purified from Sf9 insect cells. PCCT236 is a truncated protein which contains amino acids 1-236 of the 367 amino acid of PCCT. PCCT236 does not contain the lipid binding or phosphorylation regions of PCCT.

Anti-phosphoserine antibody (61-81000) was purchased from **Zymed laboratories Inc.** Other anti-phosphoserine antibodies (SA-221, SA-222, SA-223, SA224) were obtained from **BIOMOL Research Laboratories, Inc.**

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) (170-6516)

and HRP-goat anti-rabbit IgG (H+L) (170-6515) were purchased from **BioRad Laboratories.**

2.1.4 Buffer solutions

Hank's balance saline solution (Hank's): Hank's solution was prepared as a 10X stock solution (0.4% KCl, 8% NaCl, 0.06% KH_2PO_4, 0.09% $Na_2HPO_4 7H_2O$, and 10% D-glucose). 1X Hank's was obtained by diluting 10X Hank's with sterile distilled deionized water

Phosphate Buffer Saline solution (PBS): PBS was prepared as a 5X stock solution (0.1% KCl, 4% NaCl, 0.1% KH_2PO_4, 0.575% $Na_2HPO_4 \cdot 7H_2O$). 1X PBS was obtained by diluting 5X PBS with sterile distilled deionized water.

PBS-EDTA buffer: PBS containing 2 mM ethylene diaminetetraacetic acid (EDTA).

0.5% PBST buffer: PBS containing 0.5% Tween-20.

Malachite green assay working solution: Malachite green assay working solution was prepared as previous described (Zhou and Arthur, 1992). A 0.3 % suspension of malachite green base and 4.2% of ammonium molybdate were prepared by vigorously stirring the required amount of powder in 1.5 M HCl with a magnetic stirrer for at least 30 min. To prepare 400 ml malachite green assay working solution, 300 ml malachite green solution was mixed with 100 ml 4.2% ammonium molybdate solution for 15 min. 6.4 ml of 2% Tween-20 was added. The solution was stirred vigorously for an addition 10 min and filtered using Whatman #2 filter paper.

RNase-propidium iodide solution: 5 mg/100 ml propidium iodide, 100 mg/100 ml

sodium citrate, 100 mg/100 ml Triton100, 10 mg/100 ml RNase A.

Subcellular fractionation buffer: 50 mM Tris-HCl (pH 7.4), 250 mM sucrose, 100 mM β-glycerophosphate, 2 mM ethyleneglycol-bis-(β-amino-ethyl ether) N,N'-tetraacetic acid (EGTA), 1 μM microcystin, 100 μM aminoethylbenzenesulfonyl fluoride (AEBSF), 200 μM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin.

Microsome solubilizing buffer: 50 mM Tris-HCl (pH 7.4), 1% nonidet P-40 (NP-40), 100 mM β-glycerophosphate, 2 mM EGTA, 1μM microcystin, 100 μM AEBSF, 200 μM benzamidine, 10 μg/ml aprotinin, and 10 μg/ml leupeptin.

10 X Electrophoresis buffer: 30 g/L Tris-base, 144 g/L glycine, 10 g/L SDS.

1 X electrophoresis buffer was prepared by diluting 100 ml 10 X buffer in 900 ml DDW.

Transfer buffer: 100 ml 10 X electrophoresis buffer, 200 ml methanol and 700 ml DDW.

2.2 Experimental methods

2.2.1 Cell culture

MCF-7 (breast adenocarcinoma) cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (50 U/ml), streptomycin (50 μg/ml) and amphotericin B (0.3 μg/ml) at 37 °C in 5% CO_2/ 95% humidified air atmosphere as previously described (Lu and Arthur, 1992).

2.2.2 Cell synchronization

2.2.2.1 Synchronization at the G1/S boundary

Log-phase MCF-7 cells in 150-mm dishes were incubated with 1.5 mM hydroxyurea for 16 h. Hydroxyurea was subsequently removed by washing the cells three times with Hank's solution. The cells were incubated in 10 ml of 10% FBS supplemented medium for 13 h. Subsequently, hydroxyurea was added from a 500 mM stock solution to yield a final concentration of 1.5 mM in the medium, and the cells were incubated for 13 h in this medium. To obtain cells at different phases of the cell cycle, the cells were washed and incubated with 10% FBS supplemented DMEM and harvested at various times.

2.2.2.2 Synchronization at mitosis

Log-phase MCF-7 cells in 150 mm dishes were incubated with 1.5 mM hydroxyurea for 16 h. Hydroxyurea was subsequently removed by washing the cells three times with Hank's solution. The cells were then incubated in 10% FBS supplemented DMEM for 6 h. Nocodazole was added from a 200 μM stock solution to give a final concentration of 0.1 μg/ml. The cells were incubated for 6 h in the presence of nocodazole. Synchronized cells at different stages of the cell cycle were obtained by washing the cells, incubating in 10% FBS-supplemented DMEM and harvesting the cells at different times.

2.2.3 Flow cytometric analysis

Cells were detached with trypsin, pelleted by centrifugation at 1000 rpm for 3 min in a GLC-2 centrifuge (Sorvall). The cells were washed three times with ice-cold PBS

containing 2 mM EDTA. The cell pellets were subsequently resuspended in 0.75 ml ice-cold PBS-EDTA buffer with a Pipetteman. While mixing, 2 ml of ice-cold ethanol (95%) was added drop-wise to fix and permeabilize the cells. Samples were stored overnight at 4 °C. The fixed cells were pelleted, washed twice with 3 ml of ice-cold PBS-EDTA buffer and resuspended in 0.8 ml RNase-propidium iodide solution (see section 2.1.4). The cells were incubated for at least 1 h (maximun 32 h) at 4 °C in the dark. The cells were taken up into a syringe with a 21 gauge needle and filtered through a 41 µm mesh macroporous nylon filter.

Flow cytometric analysis was performed on a Coulter Electronics, Inc. EPICS 753 cell sorter with laser excitation set at 488 nm (500 mW). Forward *vs.* side light scatter histograms were used to gate the intact cells and eliminate debris, while peak *vs.* integrated propidium iodide derived from the fluorescence signal were used for doublet discrimination gating. Propidium iodide fluorescence was detected through a 610 nm long pass filter. Fluorescence histograms of 256 channel resolution were based on 5000 cells satisfying the light scatter and doublet gating criteria. Cell cycle determinations were performed using the PARA 1 analysis program (Coulter Electronics).

2.2.4 Determination of cell number

Cells were washed twice with Hank's solution, harvested with trypsin and transferred to a 50 ml conical tube. The cells were pelleted by centrifugation at 1000 rpm for 3 min and the pellet was resuspended in 20 ml Hank's solution. After dispersion with a 21-gauge needle, aliquots of the cell suspension were added to 10 ml isoton II solution in an acuvette. The cells were dispersed by passing through 21-gauge needle and the cell number was

determined with a model ZM Coulter counter.

2.2.5 Extraction of lipids from cells and medium

Phospholipids were extracted from the cells as previously described (Zhou and Arthur, 1995). Briefly, cell pellets were extracted once with 5 ml chloroform/methanol (1:1, v/v) and then twice with chloroform/methanol (2:1, v/v). The combined lipid extract was dried under a stream of nitrogen. Lipids were then dissolved in 8 ml chloroform/methanol (2:1, v/v), 6 ml of 0.9% potassium chloride was added and mixed by vortexing. Finally another 4 ml chloroform/methanol (2:1, v/v) was added and mixed thoroughly to give a biphasic system of chloroform/methanol/0.9% KCl (4:2:3, v/v/v). The lipid extract was left at room temperature to allow phase separation. The water and organic phases were completely separated by centrifugation at 1000 rpm for 3 min in a GLC-2 centrifuge (Sorvall). The upper water phase was removed while the lower chloroform phases containing phospholipids were stored at -20 °C for lipid analysis.

For all the radioactive incorporation experiments, lipids were extracted by the following procedure due to the low cell number. The cell pellet was extracted with 2 ml chloroform/methanol (2:1), vortexed and left at -20 °C overnight. 1 ml of 0.9% KCl was added, vortex thoroughly and left on ice for 30 min. The aqueous and organic phases were completely separated by centrifugation at 1000 rpm for 3 min. Aqueous phase and chloroform phase were stored at -20 °C.

Medium was extracted as previous described with modification (Walkey *et al.*, 1994). Briefly, 2 ml of chloroform/methanol (2:1) was added to 1 ml medium. The mixture was

vortexed thoroughly and left on ice for 30 min. The two phases were completely separated by centrifugation at 1000 rpm for 3 min. The aqueous and chloroform phases were stored at -20 °C.

2.2.6 Separation of phospholipids and neutral lipids

Lipids were separated by thin-layer chromatography (TLC) on Whatman K6 plates. TLC plates were preactivated by heating in an oven at 110 °C for 1 h. The activated TLC plates were covered with a piece of clean glass and allowed to cool to room temperature before the samples were applied. The lipid extracts were dried with nitrogen and resuspended in 100 µl chloroform/methanol (2:1). For experiments to investigate the incorporation of radioactive labelled precursors, lipid markers were added prior to the application of samples on TLC. The sample tubes were washed twice with 100 µl chloroform/methanol (2:1, v/v) and applied on the corresponding lanes. Phospholipids were separated by chromatography with chloroform/methanol/water/acetic acid (50:37.5:2:3.5, by vol.) (Zhou and Arthur, 1995) while neutral lipids were separated with chloroform/ methanol/acetic acid (98:2:1, by vol). Both phospholipids and neutral lipids were visualized by iodine staining and individual lipids were identified by comparing the R_f values to authentic standards.

2.2.7 Separation of choline-containing metabolites

The water soluble choline containing metabolites (Cho, P-Cho, CDP-Cho and GPC) in the aqueous phase of cell extract were separated by TLC as described below. The samples

were dried using a Speed Vac concentrator. The samples were then resuspended in 20 µl of markers solution (20 mM choline, 20 mM P-Cho, 20 mM CDP-Cho and 20 mM GPC) and applied on TLC. The sample tubes were washed once with 15 µl marker solution and applied on the corresponding lanes. The TLC plates were developed in CH_3OH/0.15 M $NaCl/NH_4OH$ (50:50:5, by vol.), and the metabolites were visualized by iodine staining and identified by comparing the R_f values to those of authentic standard markers (Cho $R_f = 0.206$; P-Cho $R_f = 0.500$; GPC $R_f = 0.647$; CDP-Cho $R_f = 0.766$).

Choline-containing metabolites extracted from the medium were separated by developing sequentially in two solvent systems. The TLC plate was first developed in chloroform/methanol/ acetic acid (98:2:1, by vol.). The TLC plate was left in a fume hood at room temperature overnight to allow the complete evaporation of the first solvent system. The plate was dried using a hair dryer before developing in the second solvent system. The TLC plate was subsequently developed in CH_3OH/0.15 M $NaCl/NH_4OH$ (50:50:5, by vol.), and the metabolites were visualized by iodine staining and identified by comparing the R_f values to those of authentic standards.

2.2.8 Quantitation of phospholipid

The phospholipids bands on the TLC plates were scraped into test tubes and 2 ml of 70% perchloric acid was added. The tubes were covered with teflon and placed in a block heater at 160 °C. Lipids were digested until the solution was clear. Phospholipid mass was determined by direct measurement of inorganic-phosphorus using the malachite green assay (Zhou and Arthur, 1992). Briefly, 100 µl lipid digests were transferred into test tubes

(triplicate measurement) with 300 µl distilled deionized water (DDW). Perchloric acid alone was digested and used for preparation of standard curves. To prepare standard curves, standard phosphate solution (10-1000 ng) were pipetted into test tubes with 100 µl digested perchloric acid. DDW was added to bring the volume to 400 µl. After mixing, 2 ml of freshly-prepared 0.3% malachite green working solution was added to both the standard and sample tubes followed by immediate vortexing. The tubes were left at room temperature for 20 min before measuring the absorbance at 660 nm in a Hitachi U-2000 double-beam spectrophotometer with DDW as reference. ΔOD was obtained by subtracting the blank OD (digests of corresponding silica gel without lipid) from the sample OD values. The quantities of phospholipids were calculated from the standard curves generated.

2.2.9 Measurement of cellular phospholipid content at different stages of the cell cycle

Exponentially growing MCF-7 cells in 150 mm dishes were synchronized at the G1/S boundary or mitosis as described in section 2.2.2. Synchronized cells at different stages of the cell cycle were obtained by harvesting cells at various times after removal of the synchronizing agent. At each time point, cells in four dishes were harvested with trypsin and combined. The cells were washed once with Hank's solution and resuspended in 10 ml Hank's solution. The cell suspension was passed through a 21 gauge needle. 50 µl aliquots were taken to determine the cell number using a model ZM coulter counter. Another sample was processed for flow cytometric analysis to establish the cell cycle distribution of the cells. Lipids were extracted from the rest of the cells, separated on TLC and quantitated by the malachite green assay (section 2.2.8).

2.2.10 Time-course of incorporation of [^{14}C]choline and [^{14}C]ethanolamine into phospholipids

Exponentially growing MCF-7 cells were plated in 60 mm dishes and incubated for two days. The cells were incubated with medium containing [^{14}C]choline ($1.62x10^6$ dpm/dish) or [^{14}C]ethanolamine ($1.39x10^6$ dpm/dish) for 1, 3 and 5 h. At each time point, duplicate dishes of cells were harvested by trypsin. Lipids were extracted from a known number of cells and separated into individual phospholipids by TLC with a solvent system of chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol.). Radioactivity incorporated in PC and PE was determined by scintillation counting and the value were expressed as dpm/10^6 cells.

2.2.11 Incorporation of [^{3}H]choline and [^{14}C]ethanolamine in phospholipids during S and G2/M phases

MCF-7 cells in 150 mm dishes were synchronized at the G1/S boundary by the double hydroxyurea treatment. The cells were subsequently released into the cell cycle by removing the cell cycle blocking agent (section 2.2.2.1). At 0, 3, 6, and 9 h after release, the cells were incubated with medium containing [^{3}H]choline ($3.13x10^6$ dpm/dish) and [^{14}C]ethanolamine ($0.55x10^6$ dpm/dish) for 1, 2 and 3 h to study the relative rates of incorporation of label into PC and PE at different stages of S and G2/M phases. At each time point, duplicate dishes of cells were washed three times with ice-cold Hank's solution and harvested by detachment with trypsin. The cell pellets were washed once with Hank's solution and resuspended in 10 ml Hank's solution. Aliquots were taken to determine the

cell number, while the rest of cells were extracted using a biphasic solvent system as described. The aqueous phase containing water soluble metabolites was separated on TLC by CH_3OH/0.15 M NaCl/NH_4OH (50: 50: 0.5, by vol.). While the chloroform phase containing phospholipids were separated by chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol.). Radioactivity incorporated in PC, PE, as well as Cho, P-Cho and GPC was determined by scintillation counting and expressed relative to the cell number.

2.2.12 Time-course of incorporation of [methyl-^{3}H]lysoPC into phospholipids

Exponentially growing MCF-7 cells in 60 mm dishes were incubated in 2 ml medium containing [methyl-^{3}H]lysoPC (1.51 x 10^6 dpm/dish) for 1, 2, 3, 5, 7, 10 h. At each time point, the labelled medium was removed and the cells were washed three times with Hank's solution and harvested by detachment with trypsin. Lipids were extracted from a known number of cells (section 2.2.5). The aqueous and chloroform phases of the cell extracts were separated by TLC and the plates were stained with iodine to visualize the phospholipids and the choline containing metabolites (section 2.2.6 and section 2.2.7). Radioactivity incorporated in lysoPC, SM, PC, and PE, as well as Cho, P-Cho and GPC fractions were determined by scintillation counting.

Lipids were also extracted from the medium using a biphasic system (section 2.2.5). To determine the radioactivity associated with lysoPC in the medium, 100 µl chloroform phase of medium extract was dried and the associated radioactivity was determined by scintillation counting. The aqueous phase of the medium extract were separated by TLC (section 2.2.7). The radioactivity incorporated in Cho, P-Cho, and GPC was determined by

scintillation counting.

2.2.13 Incorporation of [methyl-^{3}H]lysoPC and [1-palmitoyl-^{14}C]lysoPC into phospholipids

Exponentially growing cells in 60 mm dishes were incubated in 2 ml medium containing [methyl-^{3}H]lysoPC (1.05×10^{6} dpm/dish) and [1-palmitoyl-^{14}C]lysoPC (1.5×10^{6} dpm/dish) for 30, 60, 90, and 120 min. At various times, duplicate dishes of cells were washed three times with Hank's solution and harvested by detachment with trypsin. Lipids were extracted from the medium and the cells (section 2.2.5). The lipid metabolites in the aqueous phase of the extracts were separated by TLC. Radioactivity incorporated in Cho, P-Cho, and GPC was determined by scintillation counting. While the radioactivity associated with the fatty acyl-CoA fraction was determined by measurement of the radioactivity associated with 100 µl aqueous phase by scintillation counting. Phospholipids and neutral lipids in chloroform phases of the lipid extracts were separated by TLC (section 2.2.6). Radioactivity incorporated in lysoPC, PC, SM, PE, as well as monoacylglycerol (MAG), fatty acid (FA), diacylglycerol(DAG) and triacylglycerol (TAG) fractions were determined by scintillation counting.

2.2.14 Phosphatidylcholine catabolism in proliferating cells

MCF-7 cells in 60 mm dishes were incubated in 2 ml medium with [methyl-^{3}H]lysoPC (5.133×10^{5} dpm/dish) and [1-palmitoyl-^{14}C]lysoPC (1.131×10^{5} dpm/dish) for 3 h to label PC in cell. The medium was removed and cells were washed three times with

PBS + 1% BSA. The cells were incubated in 2 ml medium without label precursors for an addition 2 h. At time 0, the cells were washed three times with PBS + 1% BSA and incubated with 2 ml 10% FBS-supplemented medium for 2, 4, 6, 8, 10, 12 h. At each time point, the medium was removed and the cells were washed three times with Hank's buffer before harvesting by trypsin. Lipids were extracted from both the medium and the cell and the individual component were separated by TLC. Radioactivity incorporated in lysoPC, PC, SM, and PE, as well as MAG, DAG, FA, and TAG were determined by scintillation counting. The loss of radioactivity associated with PC in cells extracts were monitored as an indicator of PC degradation. Choline-containing metabolites in aqueous phase were also separated by TLC. Radioactivity incorporated in Cho, P-Cho, GPC, and CDP-Cho fractions were determined by scintillation counting.

2.2.15 PC catabolism during S and G2/M phases

MCF-7 cells in 60 mm dishes were synchronized at the G1/S boundary with hydroxyurea (section 2.2.2.1). Three hours before the end of synchronization, the cells were labelled with [methyl-^{3}H]lysoPC in the presence of hydroxyurea (to continuously block cells at the G1/S boundary). At the end of incubation, the media were removed and the cells were washed three times with Hank's solution. Subsequently, the cells were chased in 2 ml of 10% FBS-supplemented medium. At selected intervals, the medium was removed and the cells were washed three times with Hank's solution before harvesting by trypsin. Lipids were extracted from the medium and the cells (section 2.2.5). Phospholipids as well as choline-containing metabolites were separated by TLC. Radioactivity incorporated in PC, lysoPC,

SM, PE, Cho, P-Cho, and GPC fractions were determined by scintillation counting.

2.2.16 PC catabolism during S and G2/M phases.

MCF-7 cells in 60 mm dishes were synchronized at the G1/S boundary by the hydroxyurea treatment as described previously (section 2.2.2.1). Five hours before the end of synchronization, the cells were incubated with [methyl-^{3}H]lysoPC (2.068×10^5 dpm/dish) for 3 h in the presence of hydroxyurea to label PC in cells. The medium was removed and the cells were washed three times with Hank's solution. The cells were incubated with FBS-supplemented medium with hydroxyurea but without [methyl-^{3}H]lysoPC for 2 h. At time 0, the cells were washed three times with PBS + 1% BSA and subsequently incubated in medium without hydroxyurea (to allow cell progression along the cell cycle) to study PC catabolism during S and G2/M phases.

A separate experiment was performed to confirm PC catabolism during G2/M phase. Exponentially growing cells in 60 mm dishes were synchronized at the G1/S boundary by the hydroxyurea treatment (section 2.2.2.1) and subsequently released into the cell cycle. The cells were labelled with [methyl-^{3}H]lysoPC (3.778×10^5 dpm/dish) for 3 h (between 1 ~ 4 h post the G1/S blockage). The medium was removed and the cells were washed three times with PBS + 1% BSA. The cells were then incubated in 10% FBS-supplemented medium for 2 h (between 4 ~ 6 h post the G1/S blockage) before they were chased in G2/M phase.

At selected intervals, medium was removed, the cells were washed three times with Hank's solution before harvesting by trypsin. Lipids were extracted from both medium and

cells using a biphasic system. Phospholipids as well as choline-containing metabolites were separated by TLC as described previously. The radioactivity incorporated in PC, lysoPC, SM, PE, Cho, P-Cho, and GPC fractions were determined by scintillation counting.

2.2.17 Preparation of subcellular fractions

Synchronized cells in 150 mm dishes were washed twice with ice-cold Hank's solution and harvested by detaching with trypsin. The cell pellets were obtained by centrifugation at 1000 rpm for 3 min and resuspended in 1 ml subcellular fractionation buffer (section 2.1.4). The cells were disrupted by sonication with 4 bursts of 10 s each at a power setting of 7. Subcellular fractions were isolated by differential centrifugation as described below. Briefly, the samples were centrifuged at 15,500 rpm (19600 x g) (Beckman J2-HS centrifuge with a JA 21 rotor) for 10 minutes at 4 °C to remove unbroken cells, cell debris, nuclei, as well as the mitochondria fraction. The supernatant was centrifuged at 50,000 rpm (229600 x g_{max}) in a Ti70.1 rotor for 30 min at 4 °C. The resulting supernatant (cytosolic fraction) was stored at -20 °C while the pellet (microsomal fraction) was rinsed once with subcellular fractionation buffer, homogenized in microsome solubilizing buffer (section 2.1.4) with a Dounce homogenizer pestle A and stored at -20 °C. The protein content of each fraction was determined by the method of Lowry *et al* (Lowry *et al.*, 1951).

2.2.18 Determination of enzyme activities

CTP:phosphocholine cytidylyltransferase assay

CTP:phosphocholine cytidylyltransferase (PCCT) activity was assayed at 37 °C for

30 min according to Tercé, *et al* (Tercé, *et al.*, 1988) with 50 μg microsomal protein. The reaction mixture (250 μl) contained 20 mM Tris-HCl, pH 7.8, 6 mM $MgCl_2$, 8 mM CTP, and 4 mM [^{14}C]P-Cho (0.1μCi/μmol). The reaction was initiated by the addition of 50 μg microsomal protein and terminated by boiling the reaction mixture for 3 min. CDP-Cho (R_f: 0.73) was separated from P-Cho (R_f: 0.48) by TLC with methanol:0.15 M NaCl:28% NH_4OH (50:50:5, by vol.) as the developing solvent. CDP-Cho was identified by iodine staining and the incorporated radioactivity was determined by scintillation counting.

CTP: phosphoethanolamine cytidylyltransferase assay

CTP: phosphoethanolamine cytidylyltransferase (PECT) activity was determined according to the method of Sundler (Sundler, 1975). The reaction mixture (300 μl) contained 20 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 1 mM P-Etn, 5 mM DTT and 2 mM [^{3}H]CTP (0.5 μCi/μmol). The reaction was initiated by the addition of 50 μg cytosolic protein and incubated at 37 °C for 30 min. The reaction was terminated by boiling for 3 min. CDP-Etn (R_f:0.48) was separated from CTP (R_f:0.15) by TLC with 1 M ammonium acetate:0.01 M EDTA/90% Ethanol (30:70, by vol.) as the developing solvent. CDP-Etn was visualized by spraying with 1% ninhydrin in acetone and followed by heating at 60 °C for 30 min. The incorporated radioactivity was determined by scintillation counting.

CDP-Cho:DAG cholinephosphotransferase assay

CDP-Cho:DAG cholinephosphotransferase (CPT) activity was assayed at 37 °C for 30 min with 50 μg microsomal protein as described by Arthur and Choy (Arthur and Choy,

1984). The reaction mixture (500 µl) contained 100 mM Tris-HCl, pH 8.6, 4 mM $MgCl_2$, 0.4 mM DAG, 5 mM DTT and 0.6 mM [^{14}C]CDP-Cho (0.2 µCi/µmol). The reaction was initiated by the addition of 50 µg microsomal protein and terminated by the addition of 3 ml chloroform: methanol(2:1, by vol.). 1 ml of 0.9% KCl was added. The mixture was vortexed vigorously and left on ice for 15 min. The water phase and organic phase were completely separated by centrifugation at 1200 rpm for 3 min in a Sorvall GLC-2 centrifuge. PC was separated by TLC and the incorporated radioactivity was determined by scintillation counting.

CDP-Etn:DAG ethanolaminephosphotransferase assay

CDP-Etn:DAG ethanolaminephosphotransferase (EPT) activity was assayed with 50 µg microsomal fraction. The reaction mixture (500 µl) contained 100 mM Tris-HCl (pH 8.6), 4 mM $MgCl_2$, 0.4 mM DAG, 5 mM DTT and 0.6 mM [^{14}C]CDP-Etn (0.5 µCi/µmol). The reaction was started by the addition of 50 µg microsomal protein and terminated by the addition of 3 ml chloroform: methanol (2:1, by vol.). 1 ml of 0.9% KCl was added and the mixture was vortexed vigorously and left on ice for 15 min. The water and organic phases were separated by centrifugation at 1200 rpm for 3 min. PE was separated by TLC and the radioactivity incorporated was determined by scintillation counting.

2.2.19 Determination of CTP pools in the cell cycle

Logarithmically growing MCF-7 cells in 150 mm dishes were synchronized at the G1/S boundary by the double hydroxyurea block (section 2.2.2). Synchronized cells at

different stages of the cell cycle were obtained by harvesting the cells at various times after they were released from the G1/S boundary. The cells were resuspended in Hank's solution and an aliquot was taken to determine the cell number, while another fraction was processed for flow cytometric analysis to determine the cell cycle distribution. To determine the CTP pool, 200 µl of 10% trichloroacetic acid (TCA) was added to a known number of cell and the samples were left on ice for 30 min. The acid-soluble nucleotides were extracted by standard techniques (Tipples and McClarty, 1993). Briefly, the precipitated material were pelleted by centrifugation at 14,000 rpm for 5 min. The resulting DNA-containing pellet was discarded while the nucleotide-containing supernatant was neutralized using freshly prepared freon:tri-N-octylamine (7.81:2.19, v/v). After thorough mixing, the samples were left on ice for 30 min. The samples were centrifuged at 14,000 rpm for 1 min to separate the two phases. The aqueous phase (upper phase) containing the nucleotides was stored at 4 °C. To measure the CTP pool, 15 µl was separated by HPLC on a 12.5 cm Whatman Partisil 5 SAX HPLC column using 100% 0.55 M ammonium phosphate buffer (pH 3.4) at 1 ml/min (Tipples and McClarty, 1993). The CTP peak was identified and quantitated by monitoring the OD at 254 nm (Beckman 166 UV wavelength detector) and by comparing the absorbance and retention times to that of authentic CTP standard. All data were plotted and processed with IBM PC50 and Beckman System Gold software.

2.2.20 Immunoblotting of PCCT

The protein samples obtained from subcellular fractionation were mixed with 5X Laemmli sample buffer. Samples were boiled for 3 min and centrifuged for 10 second. 10

μg proteins were separated on 10% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membrane. The nitrocellulose membrane was incubated with 6% nonfat milk in 0.1% PBST for 2 h at room temperature or overnight at 4 °C. The membrane was then incubated with PCCT antibody (1:5000) in 0.1% PBST with 6% milk for 1 h at room temperature with shaking. After washing four times with 0.1% PBST each time for 5 min, the membrane was then incubated with horseradish peroxidase-conjugated goat anti rabbit IgG (1:5000) for 1 h in 0.1% PBST with 6% milk. The membrane was washed four times with 0.1% PBST each time for 5 min. Finally, the membrane was incubated in the ECL detection reagents for 1 min and exposed to X-ray film.

2.2.21 Quantitation of cellular proteins

The protein content of the cellular fractions was determined by the method of Lowry (Lowry *et al.*, 1951) with BSA as standard.

3 RESULTS

3.1 Cell synchronization

Exponentially growing MCF-7 cells are randomly distributed at different stages of the cell cycle. As indicated by flow cytometric analysis, the percentage of exponentially growing MCF-7 cells at G1, S, and G2/M phases are approximately 55%, 25% and 20%, respectively (Figure 20A). To investigate the coordination and regulation of phospholipid metabolism with the cell cycle, large quantities of synchronized cells at different stages of the cell cycle are required. To obtain the necessary quantities of synchronized cells at different stages of the cell cycle, our approach was to use cell cycle inhibitors to reversibly block cells at specific points of the cell cycle. Subsequent removal of the cell cycle inhibitors was expected to generate synchronized cells that progressed along the cell cycle. Synchronized cells at different stages of the cell cycle could then be obtained by harvesting cells at various times after removal of the cell cycle block.

The suitability of a number of synchronization procedures to generate large quantities of synchronized cells at different stages of the cell cycle were investigated. These included serum starvation (Krek and DeCaprio, 1995; Northwood *et al.*, 1999), excess thymidine (Bootsma *et al.*, 1964), hydroxyurea (Adams and Lindsay, 1967; Skoog and Nordenskjöld, 1971), mimosine (Lalande *et al.*, 1990), and aphidicolin (Hubermann, 1981) treatment. Our results revealed that none of the above procedures satisfied our requirements, either because treatment of MCF-7 cells with the reagents did not yield a high population of synchronized

cells, or removal of the inhibitor did not generate synchronized cell movement (data not shown). We therefore developed two procedures to generate populations of synchronized cells required for our studies. One of these, which involves exposing the cells twice to hydroxyurea during the procedure and is therefore called the double hydroxyurea treatment, synchronizes cells at the G1/S boundary. Briefly, exponentially growing cells which had a cell cycle distribution of 55%, 25% and 20% in G1, S and G2/M phase (Figure 20A) were treated with 1.5 mM hydroxyurea (Adams and Lindsay, 1967) for 16 h. At the end of the first hydroxyurea treatment, the population of cells at G1, S, and G2/M phases were 70%, 25% and 5% respectively (Figure 20B). The cells were then released from the first hydroxyurea block into 10% FBS-supplemented medium and incubated for 13 h. Flow cytometric analysis at the end of this period showed that the majority of the cells (65%) progress through S phase into G2/M phase (Figure 20C). Finally, the cells were incubated again with 1.5 mM hydroxyurea in 10% FBS-supplemented medium for 13 h. This resulted in a distribution of approximately 90% of the cells at the G1/S boundary (G1/S boundary : S : G2/M = 90% : 5% : 5%) (Figure 20D). Release of synchronized cells from the second hydroxyurea block allows more than 80% of the cells to progress in synchrony along the cell cycle as demonstrated by flow cytometric analysis (Figure 21 and 22).

Flow cytometric analysis of synchronized cells released from the G1/S boundary revealed that the total cell cycle duration of MCF-7 cells, was approximately 21 hours. The S, G2/M and G1 phases were approximately 5.5, 5 and 10.5 hours respectively (Figure 22).

The second synchronization procedure developed, produced synchronized M phase cells. Exponentially growing MCF-7 cells were incubated with 1.5 mM hydroxyurea in

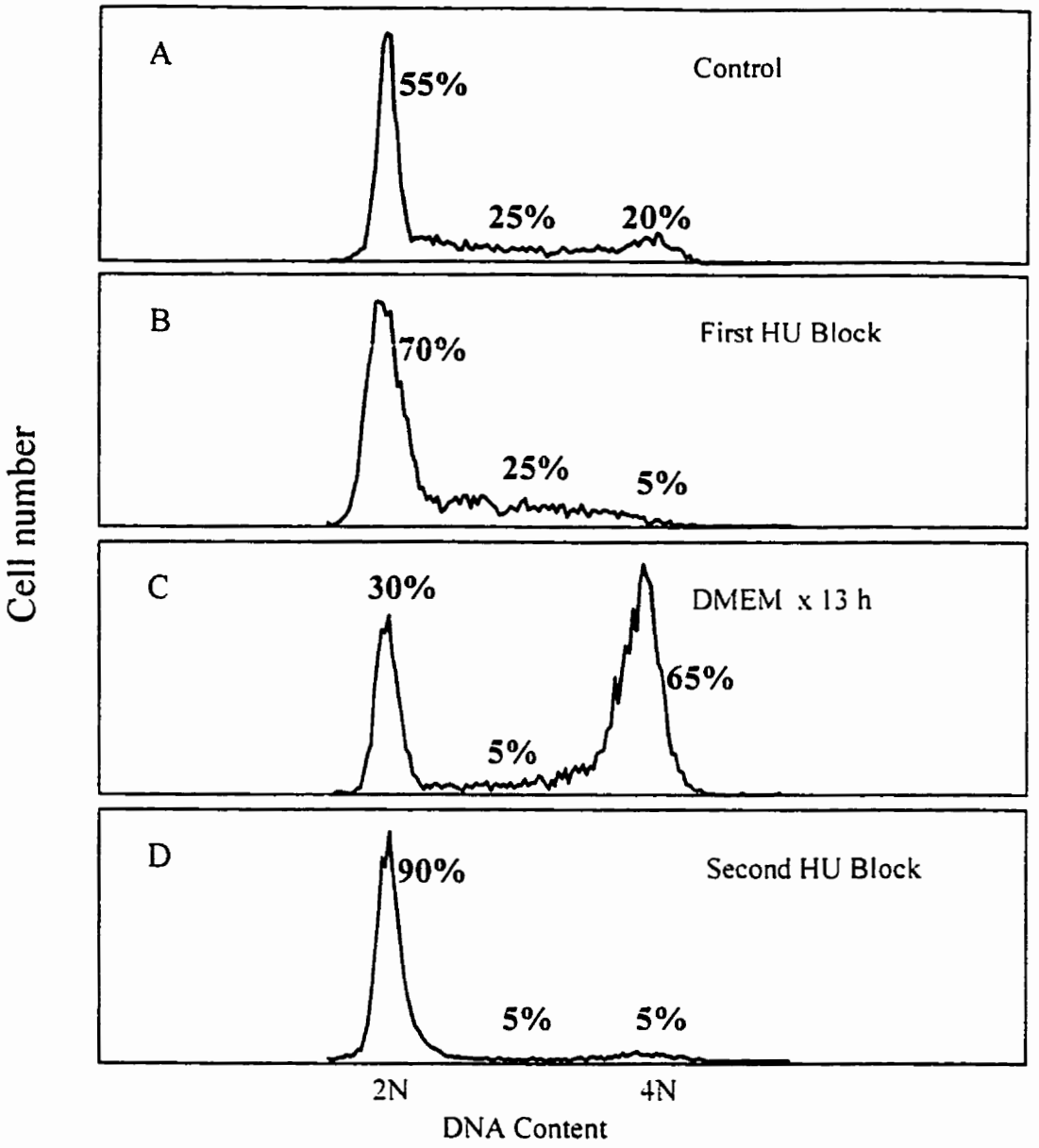


Figure 20. **Synchronization of MCF-7 cells at the G1/S boundary.** Exponentially growing cells (A) were incubated with 1.5 mM HU for 16 h (B). The cells were then incubated in growth medium for 13 h (C) followed by incubation with 1.5 mM HU for 13 h to synchronize cells at the G1/S boundary (D). The cells were harvested with trypsin, permeabilized in 70% ethanol and stained with propidium iodide. Cell cycle distribution was measured by flow cytometry.

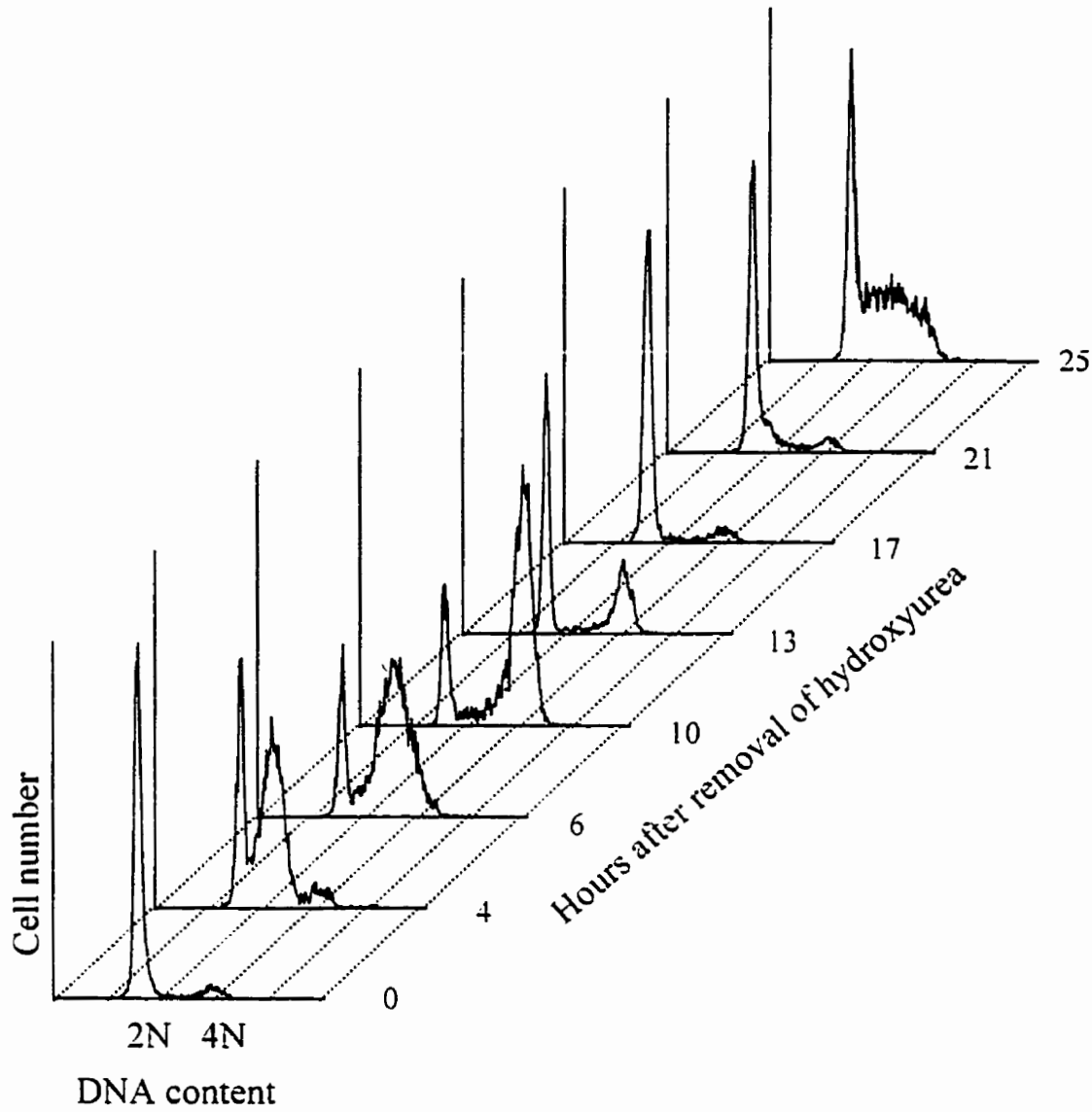


Figure 21. **Synchronized movement of MCF-7 cells along the cell cycle.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment as described in the legend of figure 20. Synchronized cells at different stages of the cell cycle were obtained by harvesting cells at various times after they were released from the G1/S boundary. Cell cycle distribution was determined by flow cytometry.

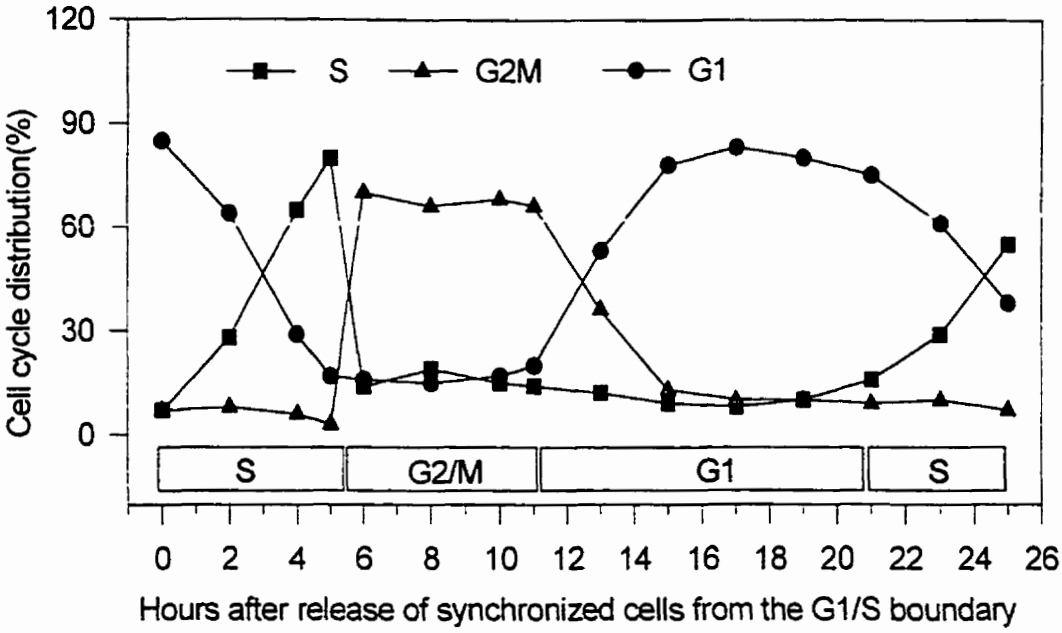


Figure 22. **MCF-7 cell cycle profile.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment as described in section 2.2.2.1. The cells were subsequently incubated in 10% FBS-supplemented medium without hydroxyurea and harvested at various times as indicated. Cellular membrane were permeabilized in 70% ethanol and incubated with propidium iodide to stain DNA. Cell cycle distribution was determined by flow cytometry. The data are average of three separate experiments.

medium for 16 h. The cells were then incubated with 10% FBS-supplemented medium for 6 h followed by incubation with nocodazole (0.1 μg/ml) for 6 h. Analysis of the cell cycle distribution at each stages of the procedure are shown in Figure 23. After the hydroxyurea treatment, the cell cycle profile was 73%, 23% and 4% (G1, S, and G2/M) (Figure 20B), indicating that the majority of the cells were arrested at the G1/S boundary. At the end of the incubation in FBS-supplemented medium, 55% of the cells were in S phase with 30% in G2/M phase (Figure 23C). Incubation with nocodazole resulted in 90% of the cells accumulating in G2/M phase (Figure 23D). The combination of hydroxyurea with nocodazole resulted in the reduction of the incubation period with nocodazole which is known to be toxic to cells on prolonged treatment (Zieve *et al.*, 1980). Synchronized cells at different stages of the cell cycle were then obtained by harvesting the cells at various times after the synchronized cells were released from mitosis (Figure 24).

3.2 Fluctuation of membrane phospholipid content in the cell cycle

Cells must double their phospholipid mass prior to cell division in mitosis so that the daughter cells formed have a similar membrane composition as the parent. However, the modality of membrane phospholipid accumulation within the cell cycle remain largely unknown (Jackowski, 1994 and 1996).

To investigate how phospholipid accumulation is coordinated with the cell cycle, exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment (section 2.2.2.1). Synchronized cells at different stages of the cell cycle were obtained by harvesting the cells at various times after they were released from the

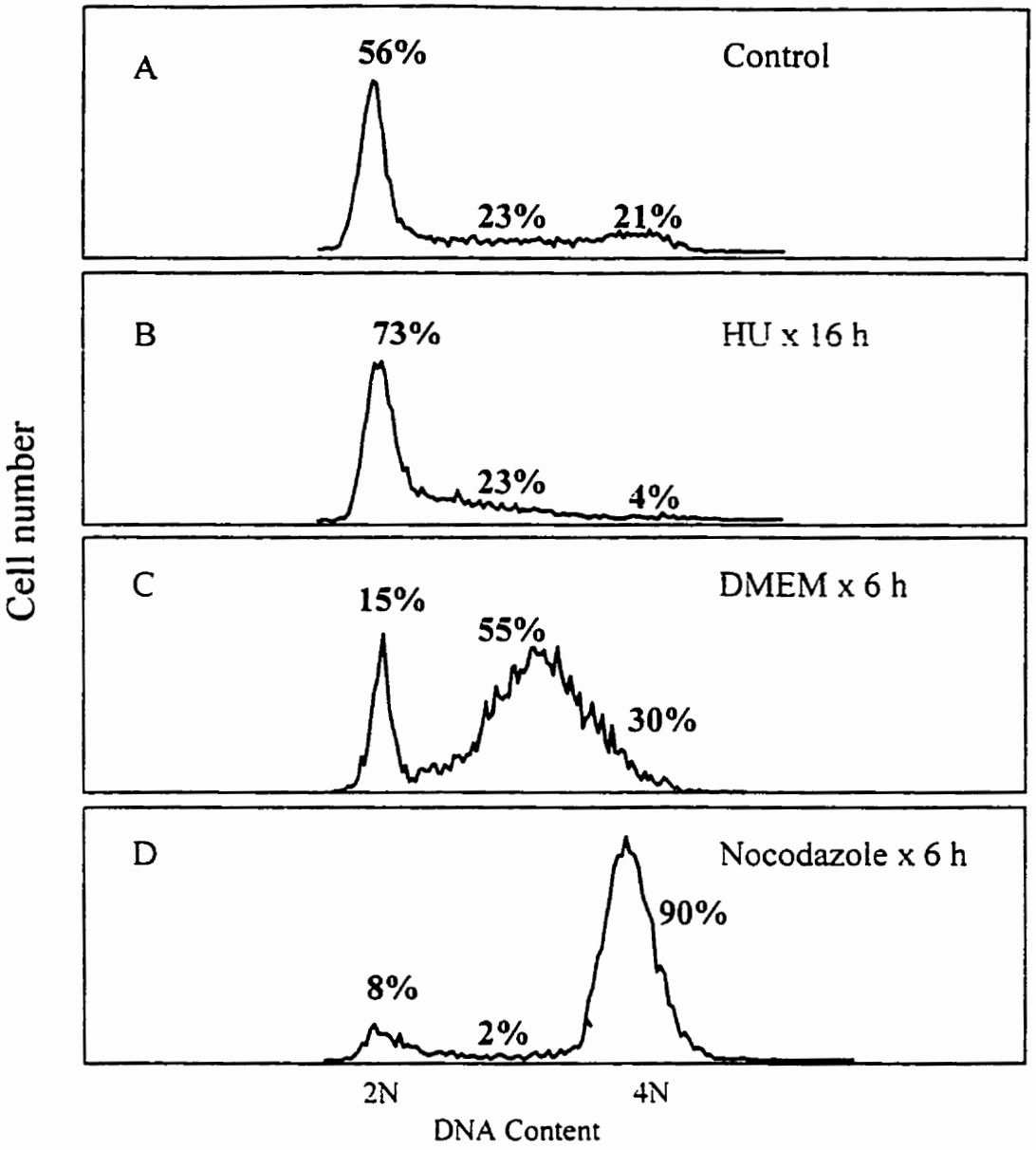


Figure 23. **Synchronization of MCF-7 cells at mitosis.** Exponentially growing MCF-7 cells (A) were incubated with 1.5 mM HU for 16 h (B). The cells were then incubated for 6 h in 10% FBS-supplemented medium (C) and subsequently incubated with 0.1μg/ml nocodazole for 6 h to synchronize the cells in mitosis (D). The cells were harvested by trypsin, permeabilized in 70% ethanol and stained with propidium iodide. Cell cycle distribution was measured by flow cytometry.

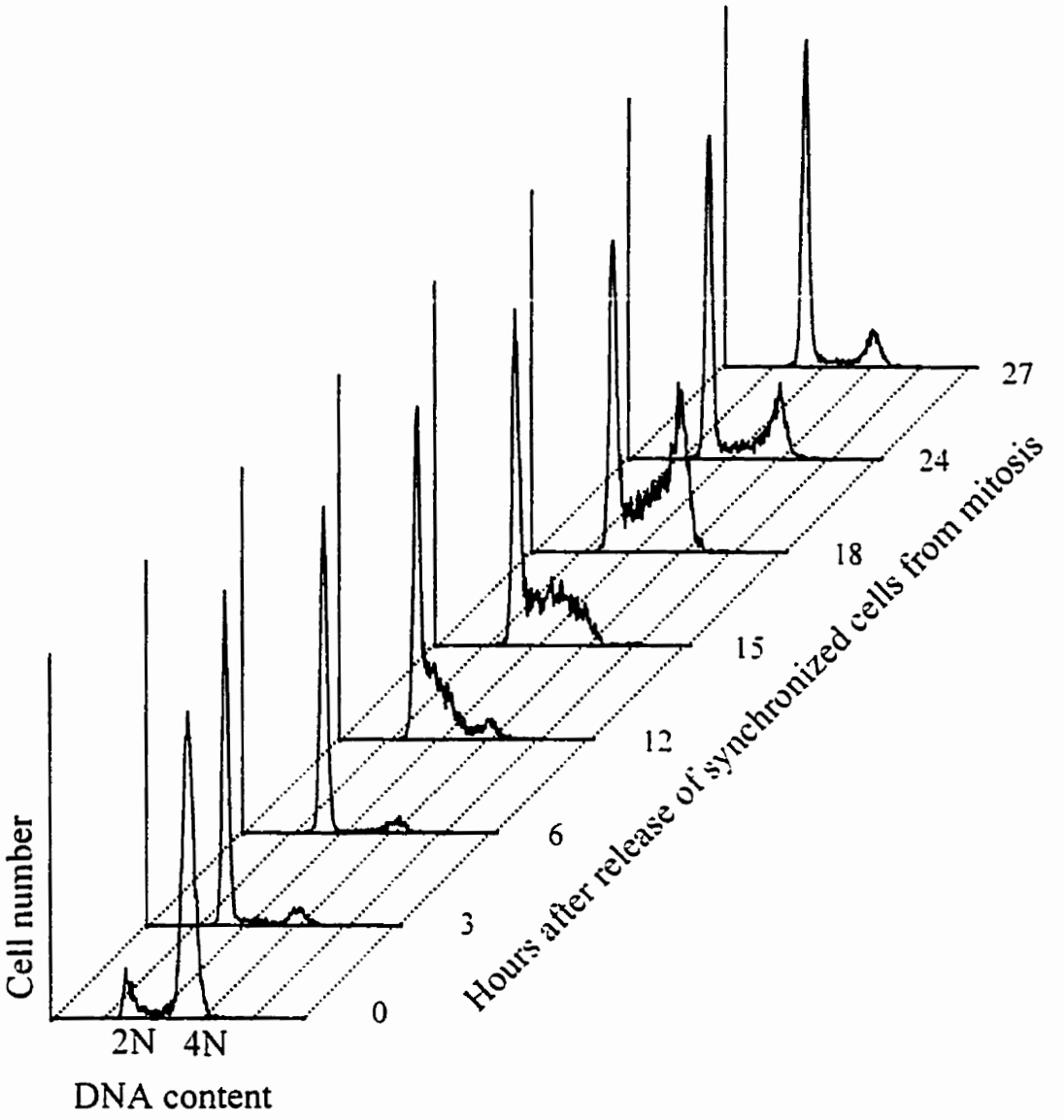


Figure 24. **Synchronized movement of MCF-7 cells along the cell cycle.** Exponentially growing MCF-7 cells were synchronized in mitosis as described in the legend of Figure 23. Synchronized cells at different stages of the cell cycle were obtained by harvesting the cells at various time after they were released from mitosis. Cell cycle distribution was determined by flow cytometry.

G1/S boundary. Measurement of phospholipid content along the cell cycle revealed that the levels of PC, PE and PI decreased 25.8%, 22.5% and 19.3% respectively, during the S phase which was followed by an increase of 78.6%, 49.2% and 53.9% respectively, in the G2/M phase (Figure 25). There was a 20.0%, 24.1% and 23.0% decrease in the mass of PC, PE, and PI respectively as cells entered the G1 phase presumably as a consequence of cell division (Figure 25). Analysis of the relative composition of PC, PE, and PI at different phases of the cell cycle are displayed in Table 4A. These results show that the content of the three phospholipids relative to each other did not change significantly along the cell cycle (Table 4A).

To demonstrate that the observed changes in cellular PC, PE and PI levels were a true cell cycle dependent event and also exclude the possibility that they were caused by hydroxyurea, the cell synchronization agent. MCF-7 cells were synchronized in mitosis by the sequential treatment of the cells with hydroxyurea and nocodazole (section 2.2.2.2). Synchronized cells at different stages of the cell cycle were obtained by harvesting cells at different times following their release from mitosis. Determination of the phospholipids mass showed that the levels of PC, PE, and PI decreased 34.8%, 34.0% and 32.9% respectively within the first 3 h after the cells were released from mitosis (Figure 26). The phospholipid levels increase slightly in late G1 phase followed by a slight decrease in S phase. The levels of PC, PE, and PI increased 43.8%, 50.3% and 36.2% respectively between 14 to 21 h after the cells were released from mitosis which corresponds to the G2/M phase (Figure 26). Thus, the cell cycle dependent fluctuation of cellular PC, PE, and PI levels in cells synchronized in mitosis showed a similar pattern with that of cells initially

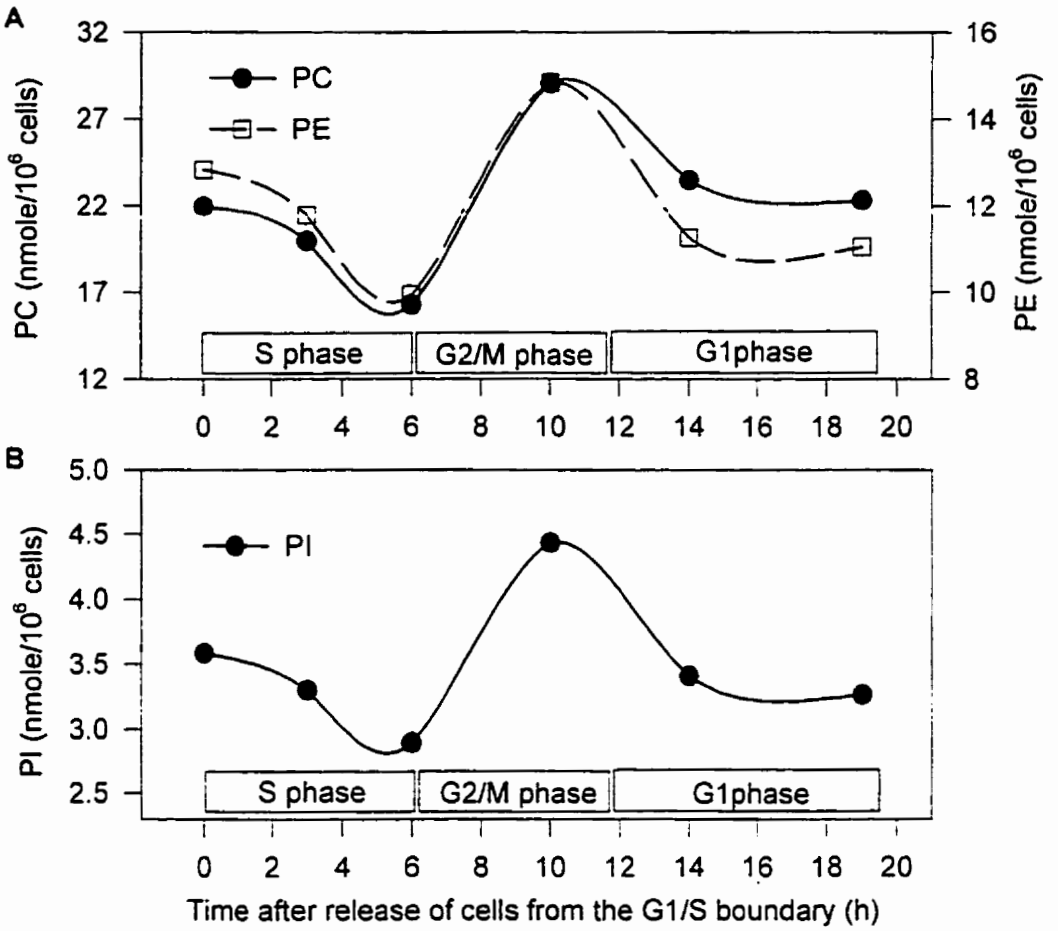


Figure 25. **Membrane phospholipid fluctuation during the MCF-7 cell cycle.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment and subsequently released into the cell cycle (section 2.2.2). At the selected interval, the cells were harvested by trypsin. Lipids were extracted from a known number of cells and separated by TLC. PC, PE (A) and PI (B) were quantitated by direct measurement of lipid-phosphorus as described in section 2.2.8. The data are representative example of four separate experiments.

Table 4. **Relative membrane phospholipid composition at different stages of the cell cycle.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary (A) or mitosis (B) as described in section 2.2.2. Synchronized cells at different stages of the cell cycle were obtained by harvesting cell at various times after cells released. Phospholipids were extracted, separated by TLC and quantitated by malachite green assay. Data were expressed as a percentage of the total lipid phosphorus in PC + PE + PI. The results are the means ± S.D. of three separate experiments.

A. Synchronized cells released from the G1/S boundary

Hours	Phospholipid composition (% of total)		
	PC%	PE%	PI%
0	57.2 ± 1.1	33.4 ± 2.8	9.3 ± 1.5
3	57.0 ± 3.2	33.6 ± 2.6	9.4 ± 0.5
6	55.9 ± 3.5	34.1 ± 2.3	9.9 ± 1.2
10	58.1 ± 2.1	32.7 ± 1.6	9.2 ± 1.1
14	58.6 ± 4.2	32.5 ± 1.8	8.9 ± 1.4
19	57.9 ± 2.2	33.2 ± 1.3	8.9 ± 1.7

B. Synchronized cells released from mitosis

Hours	Phospholipid composition (% of total)		
	PC%	PE%	PI%
0	58.0 ± 3.5	33.8 ± 1.7	8.3 ± 0.3
3	57.6 ± 1.2	34.0 ± 1.2	8.4 ± 0.7
7	58.1 ± 2.3	33.7 ± 1.5	8.2 ± 0.3
11	58.1 ± 2.7	34.1 ± 2.1	7.9 ± 0.8
14	58.2 ± 3.1	33.4 ± 1.9	8.4 ± 0.9
17	57.8 ± 2.9	33.8 ± 1.8	8.4 ± 1.3
21	58.1 ± 2.7	34.0 ± 1.3	7.9 ± 1.8

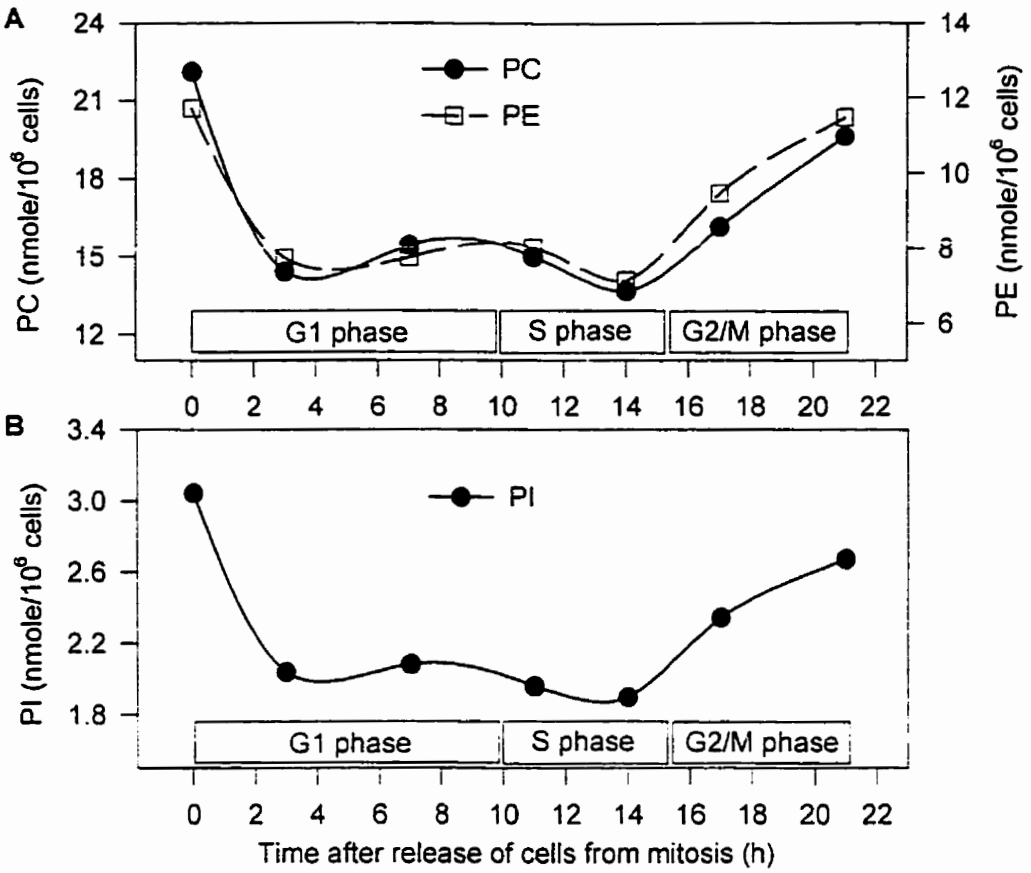


Figure 26. **Membrane phospholipid levels in synchronized cells released from mitosis.** Exponentially growing MCF-7 cells were synchronized at mitosis by sequential treatment with hydroxyurea and nocodazole and subsequently released into the cell cycle (section 2.2.2.1). At the selected intervals, the cells were harvested with trypsin. Lipids were extracted from a known number of cells and separated by TLC. PC, PE (A) and PI (B) were quantitated by direct measurement of lipid-phosphorus as described in section 2.2.8. The results were expressed as nmole/10^6 cells. The data are representative example of four separate experiments.

synchronized at the G1/S boundary (Figure 25). The proportion of PC, PE, and PI was also relatively constant along the cell cycle (Table 4B), confirming the results obtained in the studies with the G1/S synchronized cells (Table 4A).

Taken together, the above studies demonstrated that PC, PE, and PI mass significantly fluctuates during S and G2/M phases. The contents of PC, PE, and PI decrease during S phase followed by accumulation in G2/M phase. However, the composition of major phospholipids remained relatively constant along the cell cycle. Since phospholipid accumulation is the net effect of both biosynthesis and catabolism, we investigated the contribution of both biosynthesis and catabolism to the observed fluctuation of phospholipid content during the cell cycle. The focus of these studies were on the metabolism of PC and PE, the two major phospholipids found in mammalian membranes. These studies are described below.

3.3 Phospholipid biosynthesis in the cell cycle

3.3.1 Incorporation of [^{3}H]choline and [^{14}C]ethanolamine into PC and PE

PC and PE are synthesized primarily through the *de novo* pathways (Kent, 1995; Vermeulen *et al.*, 1997). Incorporation of radioactive labelled precursors, [^{3}H]choline and [^{14}C]ethanolamine, into PC and PE has been widely used to study the relative rates of PC and PE biosynthesis (George *et al.*, 1991; Hatch *et al.*, 1991). We used similar approaches to study the relative rate of PC and PE biosynthesis during the cell cycle. To establish the optimum incubation conditions with the radiolabel precursors for the studies of PC and PE biosynthesis in the cell cycle, we performed a time-course incorporation assay.

Exponentially growing MCF-7 cells in 150 mm dishes were incubated in 10 ml medium containing [^{14}C]choline (1.62×10^6 dpm/dish) or [^{14}C]ethanolamine (1.39×10^6 dpm/dish) for 1, 3, and 5 h. The cells were washed three times with Hank's solution before harvesting with trypsin. Phospholipids were extracted and separated by TLC. Radioactivity incorporated in PC and PE were determined by scintillation counting. Our studies revealed that the rates of [^{14}C]choline and [^{14}C]ethanolamine incorporation into PC and PE was relatively linear within 5 h incubation (Figure 27). We subsequently used 3 h incubation periods to study the relative rates of PC and PE biosynthesis in the cell cycle.

3.3.2 PC biosynthesis during S and G2/M phases

Changes in the phospholipid content during the cell cycle occurred mainly during S, G2/M and G1 phases of the cell cycle (Figure 25 and 26). Although there is an apparent decrease in the phospholipid content after cells exit mitosis into early G1 phase (Figure 25 and 26), this change in cellular lipid content can be attributed primarily to cell division rather than lipid metabolism. Cell division in mitosis produces two daughter cells each with half the lipid content of the parental M-phase cell. As the cell numbers do not change during the S and G2/M phases, the observed changes in lipid contents during S and G2/M phases are likely to be due to changes in lipid metabolism. We therefore focussed our studies on changes in phospholipid metabolism in the S and G2/M phases.

The relative rates of PC biosynthesis at different stages of the S and G2/M phases (early S, late S, early G2/M, and late G2/M phase) were investigated. Briefly, exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea

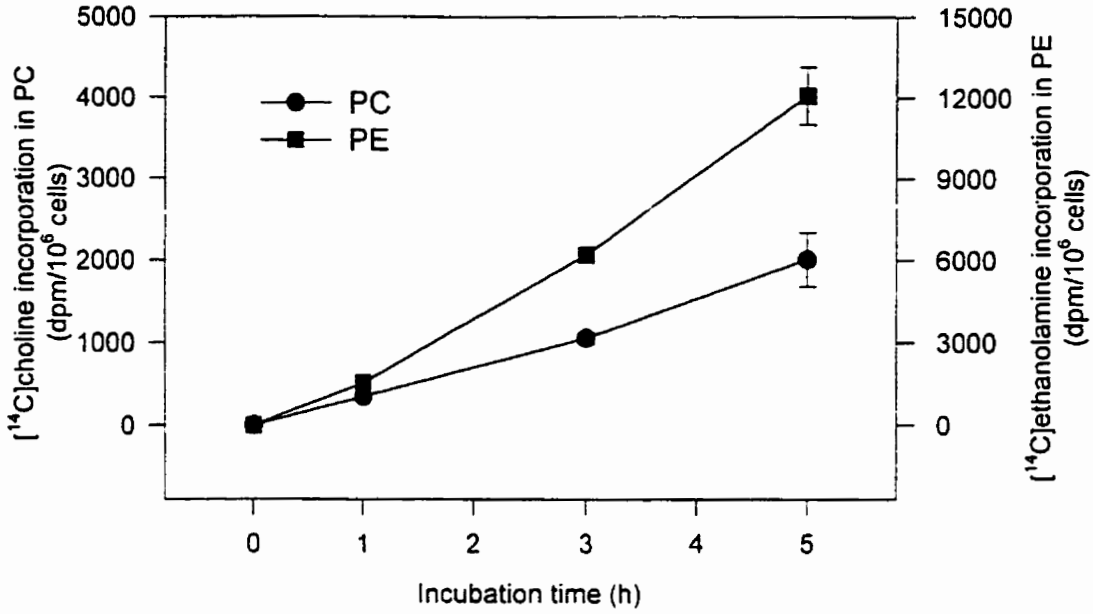


Figure 27. **Incorporation of [^{14}C]choline and [^{14}C]ethanolamine into PC or PE as a function of time.** Exponentially growing MCF-7 cells were incubated in medium containing [^{14}C]choline (1.62×10^6 dpm/dish) or [^{14}C]ethanolamine (1.39×10^6 dpm/dish) (section 2.2.10). At the indicated times, duplicated dishes of cells were washed three times with Hank's solution and harvested by trypsin. Lipids were extracted from a known number of cells and separated by TLC (section 2.2.6). Radioactivity incorporated in PC or PE were determined by scintillation counting. Results are the means ± S.E. of two separate experiments with duplicated determination.

treatment. Subsequently, the cells were allowed to progress along the cell cycle by removal of the hydroxyurea. At 0, 3, 6, and 9 h after the cells were released from the G1/S boundary, they were incubated with [^{3}H]choline for 1, 2, and 3 h. We also investigated the synthesis of PC in cells at the G1/S boundary by maintaining the presence of hydroxyurea during the incubation with [^{3}H]choline. At the end of the incubation period, the cells were harvested with trypsin. Lipids were extracted from the cells and the radioactivity incorporated into PC was determined by scintillation counting. The results of the above studies are shown in Figure 28. The rate of PC synthesis in the MCF-7 cells maintained at the G1/S boundary by continuous incubation with hydroxyurea was similar to that observed in the early S phase cells (0 - 3 h after removal of hydroxyurea) (Figure 28). This result would suggest that hydroxyurea does not affect PC synthesis in MCF-7 cells. Incorporation of [^{3}H]choline into PC in late S phase cells (3 - 6 h after removal of hydroxyurea) was greater than that observed in the early S phase cells. The results also showed greater rates of PC synthesis at all stages of the G2/M phase compared to that in late S phase. For quantitative comparison purposes, the levels of [^{3}H]choline incorporated in all phases were expressed relative to the values obtained in the G1/S synchronized cells (Table 5). The results show that the highest rates of synthesis occurred in G2/M followed by synthesis in late S phase (Table 5). These results indicate a correlation between the increase in PC biosynthesis and the observed PC accumulation in G2/M phase (Figure 25A, 26A and 28).

To investigate whether the enhanced PC biosynthesis observed above could be due to the activation of PCCT, the rate-limiting enzyme in PC synthesis, P-Cho and CDP-Cho present in the aqueous phase from the lipid extraction procedures were separated by TLC.

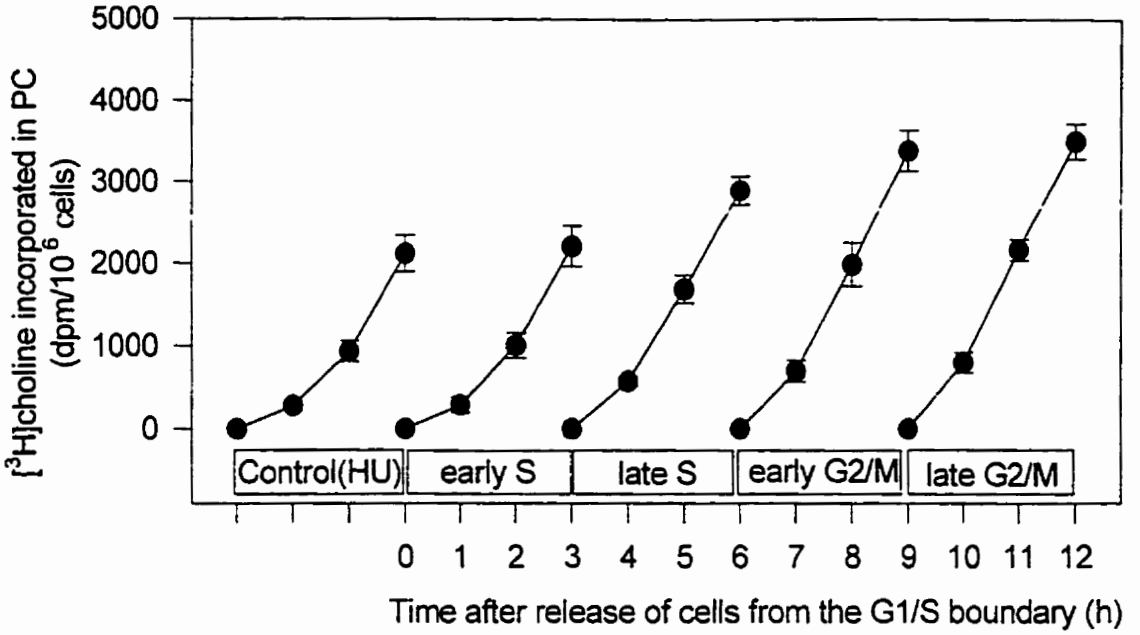


Figure 28. **Incorporation of [^{3}H]choline into PC during S and G2/M phase.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment and subsequently released into the cell cycle. At 0, 3, 6, and 9 h after release, the cells were incubated in medium containing [^{3}H]choline (3.13×10^6 dpm/dish) for 1, 2 and 3 h. In control experiments, synchronized cells were incubated with [^{3}H]choline in the presence of HU to maintain the cells at the G1/S boundary. At the indicated times, the cells were harvested by trypsin. Lipids were extracted from a known number of cells and separated by TLC. Radioactivity incorporated into PC was determined by scintillation counting. The results are the means ± S.D. of four separate experiments.

Table 5. **[^{3}H]choline incorporation into PC during S and G2/M phases.** The radioactivity incorporated into PC in Figure 28 was expressed as a percentage relative to that of the corresponding controls (+HU). The data from late S, early G2/M and late G2/M phases were compared with the corresponding data in early S phase. The results are the means ± S.D. of four independent experiments.

Time	Control (+HU)	S phase		G2/M phase	
	G1/S boundary	early S (0~3h)	late S (3~6h)	early G2/M (6~9h)	late G2/M (9~12h)
1h	100	100.0 ± 18.6	210.7 ± 53.1*	252.8 ± 68.8*	290.1 ± 64.0*
2h	100	108.3 ± 17.2	181.9 ± 30.0*	212.4 ± 22.8**	234.5 ± 40.1**
3h	100	105.0 ± 17.3	136.8 ± 9.4*	161.9 ± 27.7*	167.1 ± 23.8**

(*) $p<0.05$ compared with the corresponding label incorporation in early S phase. (**) $p<0.001$ compared with the corresponding label incorporation in early S phase (by student t-test).

The incorporated radioactivity in the two metabolites was determined by scintillation counting. The results which are displayed in Figure 29 demonstrated that relative to the levels in the control cells, radioactivity incorporated in CDP-Cho was elevated from S to G2/M phase (Figure 29A) in parallel to the increase radioactivity associated with PC (Figure 28). The radioactivity associated with P-Cho increased from early S and peaked in late S and remained relatively constant in G2/M phase (Figure 29B). The increase in radioactivity associated with CDP-Cho and PC during G2/M phase suggests that the enhanced PC biosynthesis in G2/M phase could result from the activation of PCCT (Figure 29).

3.3.3 PE biosynthesis during S and G2/M phases

Studies on the rate of PE biosynthesis in the S and G2/M phases were conducted using the approaches described above for PC but with [^{14}C]ethanolamine as the radiolabelled precursor. The results of these studies which are displayed in Figure 30 showed that [^{14}C]ethanolamine incorporation into PE during S and G2/M phases was quite different from that of PC. Apart from a higher rate of [^{14}C]ethanolamine incorporation into PE in late S phase, the rates of [^{14}C]ethanolamine incorporation into PE at all the other stages of the S and G2/M phases was similar to that in the control cells (Figure 30). This was readily apparent when the levels of [^{14}C]ethanolamine incorporated into PE in the different phases were expressed relative to the levels present in cells synchronized at the G1/S boundary (Table 6). The results revealed that there was no significant differences in the rate of [^{14}C]ethanolamine incorporation into PE at the G1/S boundary and in early S phase MCF-7 cells (Table 6).

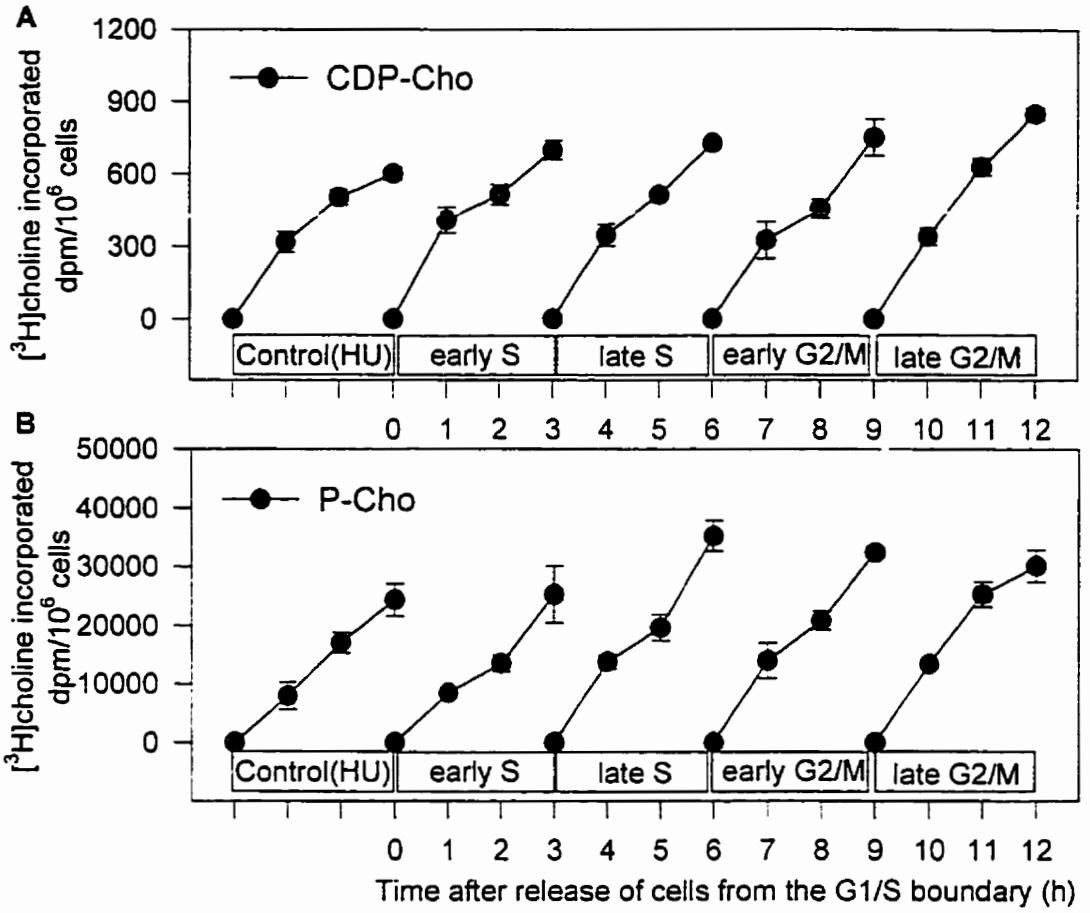


Figure 29. **Incorporation of [^{3}H]choline into CDP-Cho and P-Cho during S and G2/M phases.** The experiments were conducted as described in the legend of Figure 28. Lipids were extracted from a known number of cells using biphasic system. Aqueous phase were concentrated with a Speed Vac concentrator and separated by TLC. Radioactivity incorporated in CDP-Cho (A) and P-Cho (B) were determined by scintillation counting. The results are the means ± S.D. of four separate experiments.

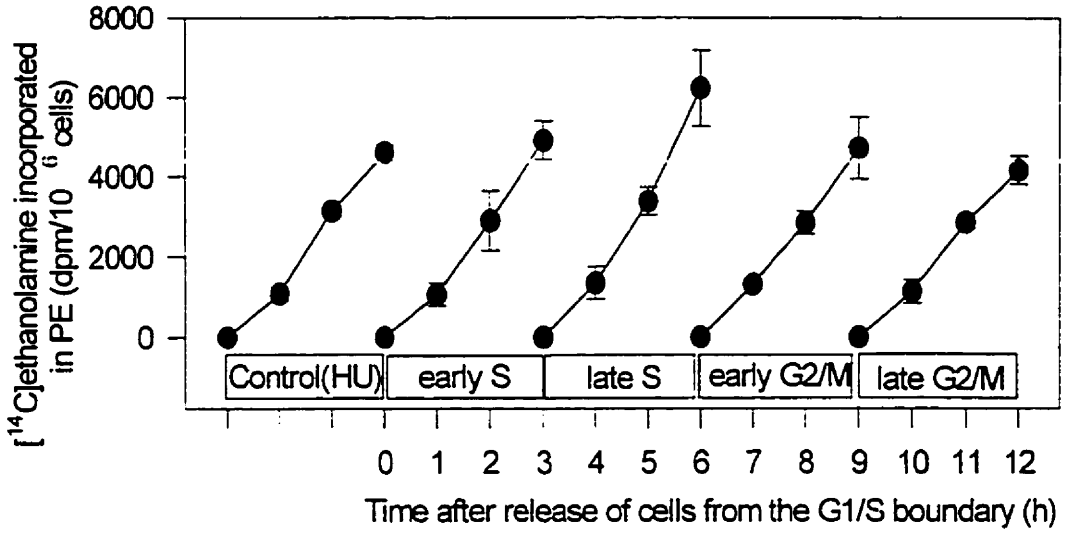


Figure 30. **Incorporation of [^{14}C]ethanolamine into PE during S and G2/M phase.** The experiments were conducted using the same protocol described for PC in the legend of figure 28 but with [^{14}C]ethanolamine (0.55×10^6 dpm/dish) as the radiolabelled precursor (section 2.2.11). At the indicated times, the cells were washed three times with Hank's solution and harvested by trypsin. Lipids were extracted from a known number of cells and separated by TLC. Radioactivity incorporated in PE was determined by scintillation counting. The results are the means ± S.D. of four separate experiments.

Table 6. **[^{14}C]ethanolamine incorporation into PE during S and G2/M phases.** The radioactivity incorporated into PE in figure 30 was expressed as a percentage relative to that of the corresponding controls (+HU). The data from late S, early G2/M and late G2/M phases were compared with the corresponding data in early S phase. The results are the means ± S.D. of four independent experiments.

Time	Control (+HU)	S phase		G2/M phase	
	G1/S boundary	early S (0~3h)	late S (3~6h)	early G2/M (6~9h)	late G2/M (9~12h)
1h	100	97.8 ± 17.3	122.3 ± 20.6	121.6 ± 10.4	104.0 ± 22.5
2h	100	92.4 ± 24.4	107.7 ± 12.8	90.5 ± 10.8	90.7 ± 3.2
3h	100	106.5 ± 8.9	134.7 ± 15.4*	102.7 ± 18.3	90.3 ± 6.8*

(*) $p<0.05$ compared with the corresponding label incorporation in early S phase (by student t-test)

Thus, hydroxyurea did not affect [^{14}C]ethanolamine incorporation into PE. After 3 h incubation, [^{14}C]ethanolamine incorporation into cells in early S, late S, early G2/M and late G2/M were 106.5%, 134.7%, 102.7% and 90.3%, respectively of the levels observed in the control cells (Table 6). Comparison of the levels of ethanolamine incorporation in early S with the other phases revealed that the increased incorporation observed in late S (34%), was statistically significant as was the decrease (10%) in late G2/M. These results revealed that the rate of PE biosynthesis did not correlate with the observed PE accumulation in G2/M, suggesting that the observed PE accumulation in G2/M phase may not be directly linked to enhanced synthesis.

3.3.4 Changes in the activity of phospholipid-biosynthetic enzymes in the cell cycle

The radiolabel incorporation studies described above revealed that the rate of PC and PE synthesis fluctuates during the cell cycle. The results also suggested that the enhanced PC synthesis in G2/M phase might be due to the activation of PCCT. The enzymes that catalyze the reactions leading to the synthesis of PC and PE have been well studied (Kent, 1995 and 1997; Vermenlen *et al.*, 1997). Since changes to the activities of these enzymes could be responsible for the changes in the rates of biosynthesis, we investigated the activities of key enzymes in the PC and PE *de novo* synthetic pathways during the cell cycle. MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea protocol. At selected intervals subsequent to removal of the blocking agent, the cells were harvested and subcellular fractions were prepared (section 2.2.17). The activities of PCCT, CPT, PECT, and EPT were assayed in the appropriate subcellular fraction (section 2.2.18). The

enzyme activities in subcellular fractions isolated from nonsynchronized (NS) exponentially growing cells were also determined for comparison. The results of these studies are displayed in Figure 31. For comparative purposes, the results obtained from the synchronized cells at different stages of the cell cycle were expressed as a percentage of that of the non-synchronized cell (Table 7). Our results demonstrated that the specific activity of PCCT at the G1/S boundary was 29.7% of that in fractions from the non-synchronized cells (Table 7). PCCT activity increase progressively through S and G2/M phases (Figure 31) to a levels of about 126% of the non-synchronized cells followed by a slight decline during the G1 phase to levels approaching that of the non-synchronized population (Table 7 and Figure 31). The specific activity of CPT, the enzyme that catalyzes the final step in PC biosynthesis, was 35.4% of that of the exponentially growing cells at the G1/S boundary (Table 7). The CPT activity increased progressively through S and G2/M phases to a levels of 157.5% of the non-synchronized cells in early G1 phase. This was followed by a slight decline during the mid- and late G1 phase (Table 7 and Figure 31). These results are consistent with the observed increase in [^{3}H]choline incorporation into PC during S and G2/M phases (Figure 28). The specific activity of PECT which is believed to be the rate-limiting enzyme in PE biosynthesis (Vermeulen *et al.*, 1993 and 1997) did not exhibit obvious changes during the cell cycle (Figure 31). At the G1/S boundary, PECT activity was 85.2% of that of the non-synchronized cells. This level of activity was maintained during S and G2/M phases. The enzyme activity increased to 105.8% of the control level at late G2/M and early G1 phase (Table 7). The specific activity of EPT which catalyzes the final step of PE biosynthesis was relatively low at the G1/S boundary and was 22.3% of that of

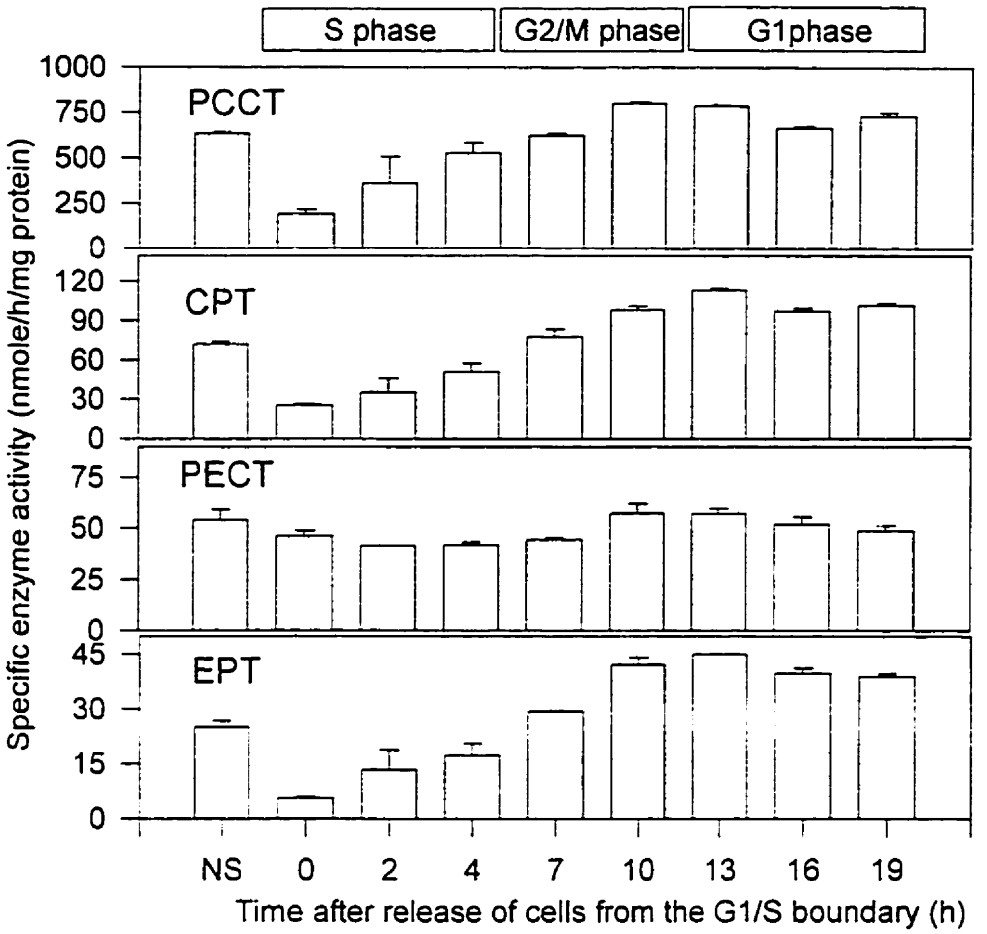


Figure 31. **Specific activity of PC and PE biosynthetic enzymes during the cell cycle.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment and subsequently released into the cell cycle. At the selected intervals, the cells were harvested. Subcellular fractions were prepared by differential centrifugation. The enzyme activity was determined as described in section 2.2.18. The enzyme activity of non-synchronized cells (NS) were also determined for comparison. The results are means ± S.D of three independent experiments.

Table 7. **The specific activity of PC and PE biosynthetic enzymes during the cell cycle relative to that of the non-synchronized cells.** The specific enzyme activities in Figure 31 were expressed as a percentage of the activity present in the non-synchronized cells (NS).

Enzyme	Control	Time after release of cells from the G1/S blockage (h)							
		0	2	4	7	10	13	16	19
	NS	G1/S	S phase		G2/M phase			G1 phase	
PCCT	100	29.7	56.9	83.1	98.3	126	124.1	105.2	115.5
CPT	100	35.4	49.9	70.7	108.1	136.5	157.5	135.3	141.2
PECT	100	85.2	76.2	77.1	82.1	105.8	105.3	96	90
EPT	100	22.3	53.4	68.9	116.9	167.8	179.2	158.5	154.8

the non-synchronized cells. However, EPT activity increased rapidly from early S to early G1 phase and began to decrease after mid- G1 phase (Figure 31 and Table 7). An increase of approximately 8-fold in EPT activity was observed between G1/S boundary and early G1 phase (Table 7).

Since the mass of cells changes during the cell cycle, the total activity of the enzymes in the cells could more appropriately explain the changes in the synthesis of phospholipids during the cell cycle than the specific activity. The total microsomal and cytosolic protein content in different stages of the cell cycle were shown on Table 8a. The total enzyme activities of key enzymes were calculated from their specific activities multiplied by the content of the subcellular protein. The results are displayed in Figure 32. The total enzyme activity of synchronized cells at different stages of the cell cycle was expressed as a percentage of that of the non-synchronized cell (Table 8b). Our result showed that at the G1/S boundary the total activities of PCCT, CPT, PECT and EPT were 35.2%, 43.6%, 87.5% and 27.7% of that of the exponentially growing cells respectively (Table 8b). PCCT activity increase progressively through S to 188.4% of the control level in the G2/M phase and declining during the G1 phase to 129.3%. CPT activity increased approximately 5-fold during S and G2/M phases to 205% at early G1 phase and declined to 159.8% at late G1 phase (Table 8b). PECT activity was increased from 87.5% at the G1/S boundary to 132.4% in the G2/M phase and declined to 107.6% in late G1 phase, while EPT activity increased from 27.7% to 254.5% during S and G2/M phase and decreased to 176% in late G1 phase (Table 8b). In general, the relative changes in the total enzyme activity were similar to that observed in comparisons of the specific activities of the enzymes (Figure 32).

Table 8a. **The contents of microsomal protein and cytosolic protein in the cell cycle.** Proliferating cells were synchronized at G1/S boundary and subsequently released into the cell cycle. Synchronized cells at different stages of the cell cycle were obtained by harvesting cells at various times after cells released. Subcellular fractions were prepared as described in the section 2.2.17. The content of microsomal and cytosolic proteins were determined by the method of Lowry (Lowry *et al.*, 1951). Results are the means of two separate experiments with duplicated determination .

Time (h)	Cell cycle stages	Microsomal protein ($\mu g/10^6$ cells)	Cytosolic protein ($\mu g/10^6$ cells)
Control	proliferating cells	107.2	110.1
0	G1/S boundary	127.1	113.1
2	Early S	114.6	112.4
4	Late S	128.1	140.4
7	G2/M	138.5	147.1
10	G2/M	160.2	137.8
13	Early G1	139.1	130.2
16	Mid G1	115.6	147.6
19	Late G1	119.9	131.6

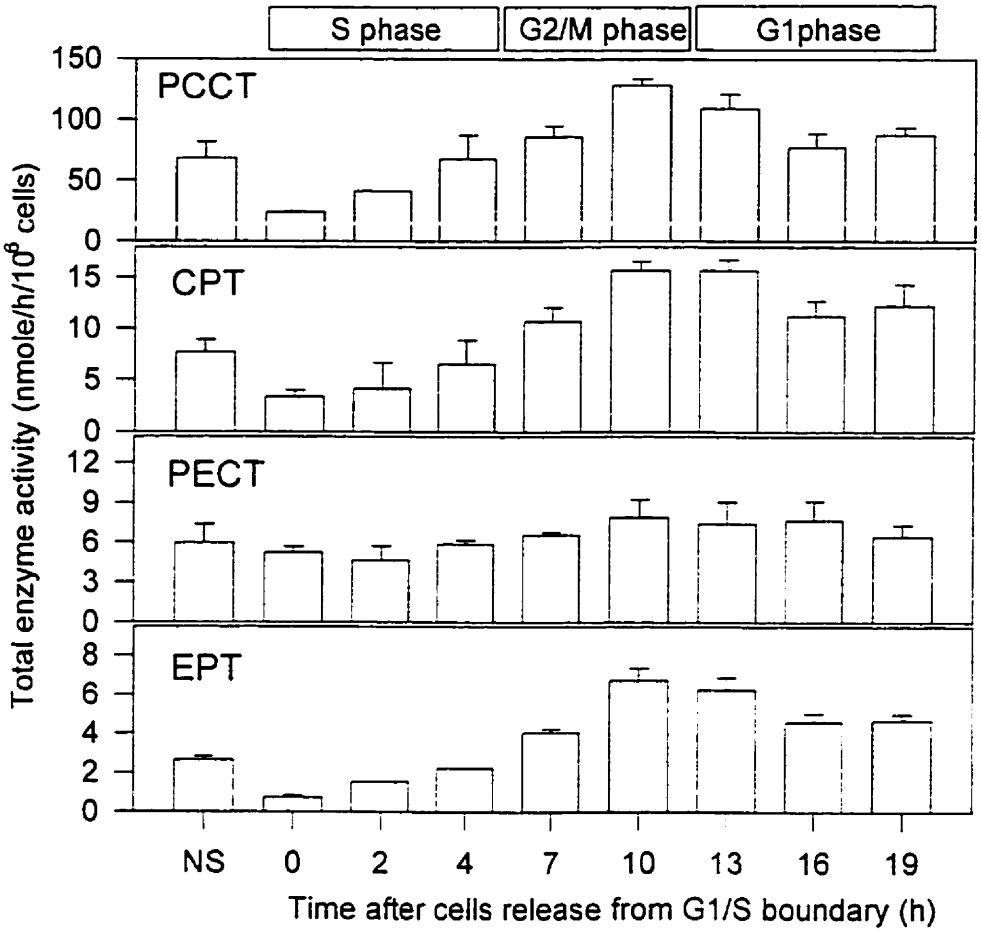


Figure 32. **Total enzyme activity of PC and PE biosynthetic enzymes during the cell cycle.** The experiments were conducted as described in the legend of figure 31. Data were expressed as nmol/h/10^6 cells to reflect the total enzyme activity in the cell. Total enzyme activity was calculated from the specific activity multiplied by the protein content in the subcellular fraction of 10^6 cells. The results are the means ± S.D. of three independent experiments.

Table 8b. **The total activity of PC and PE biosynthetic enzymes during the cell cycle relative to that of the non-synchronized cells.** The total enzyme activities in Figure 32 were expressed as a percentage of the activity present in the non-synchronized cells (NS).

Enzyme	Control	Time after release of cells from the G1/S blockage (h)							
		0	2	4	7	10	13	16	19
	NS	G1/S	S phase		G2/M phase			G1 phase	
PCCT	100	35.2	60.9	99.3	127.1	188.4	164	113.5	129.3
CPT	100	43.6	53.9	85.1	139.5	204.9	205	146.4	159.8
PECT	100	87.5	77.8	98.4	109.7	132.4	124.6	128.6	107.6
EPT	100	27.7	57.9	84	153.2	254.5	236.2	172.8	176

To exclude the possibility that hydroxyurea interferes with phospholipid biosynthesis, we assayed the enzyme activities *in vitro* in the presence and absence of hydroxyurea. Our result showed that hydroxyurea did not affect the activities of any of the enzymes examined (Figure 33).

3.3.5 Fluctuation of microsomal PCCT levels in the cell cycle

PCCT catalyzes the conversion of P-Cho to CDP-Cho, the rate-limiting step in PC biosynthesis (Kent, 1997; Tijbur *et al*, 1989). The results of our studies described in section 3.3.2 and 3.3.4 revealed that at G2/M there was an increase in [^{3}H]choline incorporation into PC and an elevation in PCCT activity. Together, these results suggest that the enhanced biosynthesis of PC in G2/M phase could be due to the enhanced activation of PCCT. PCCT exists in both particulate and soluble fractions (Vance, 1989; Kent, 1995). Activation of PCCT *in vivo* correlates with its translocation from an inactive soluble form to an active, membrane-associated species (Kent, 1997; Clement and Kent, 1999). To investigate whether the increased activity of PCCT in the cell cycle was due to increased levels of membrane-associated PCCT, immunoblotting approaches with PCCT antibodies were used to measure the PCCT levels in microsomal fractions.

Immunoblotting studies with PCCT antibody revealed that in extracts from various cell lines the PCCT antibody recognized two main bands, but in the BT549 cell line four bands were detected by the antibody (Figure 34). The major band in most of the cells lines including MCF-7 cells, had a molecular weight of 42 kD, the reported mass of purified PCCT (Weinhold *et al*., 1989; Cornell,1989a). The other band had an approximate

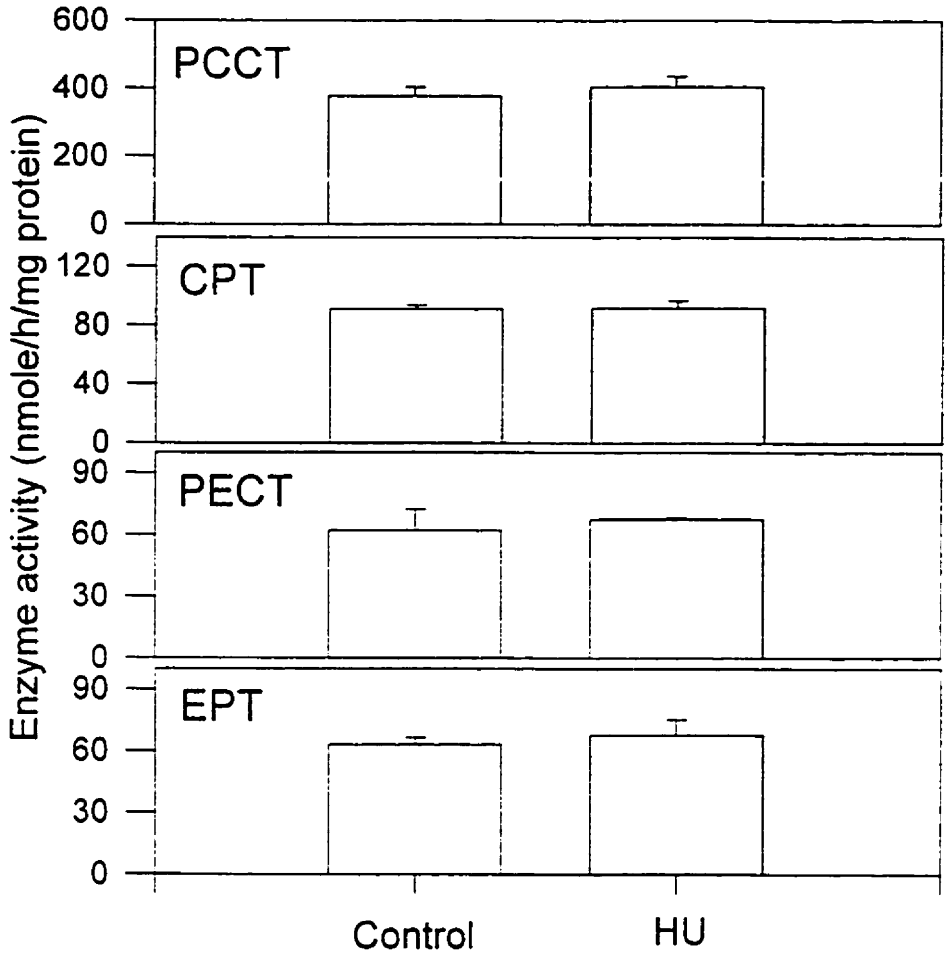


Figure 33. **Effect of hydroxyurea on the activity of PC and PE biosynthetic enzymes.** Subcellular fractions were prepared from exponentially growing MCF-7 cells. PCCT, CPT, PECT and EPT activity were assayed *in vitro* in the presence and absence of 1.5 mM HU. The results are the means ± S.D. of three separate experiments.

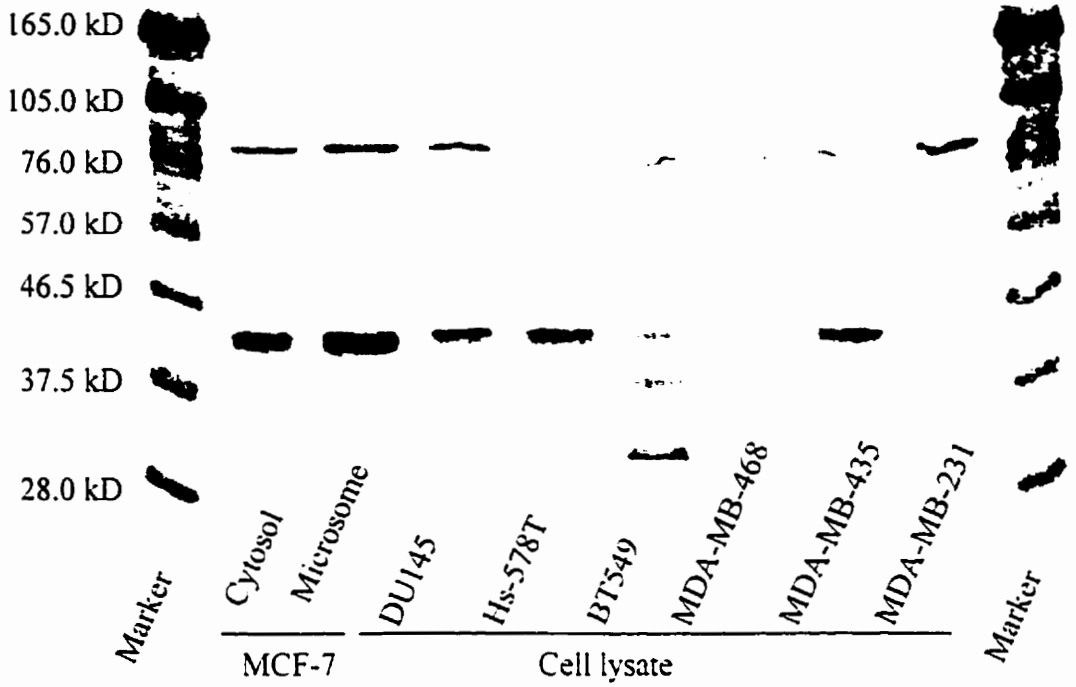


Figure 34. **Expression of PCCT in breast epithelial cancer cell lines.** 10μg of proteins from breast epithelial cancer cell lines (MCF-7, Hs-578T, BT549, MDA-MB-468, MDA-MB-435 and MDA-MB-231) and a prostate epithelial cell line (DU-145) were subjected to western blot analysis with rabbit polyclonal PCCT antibody. The samples were separated on a 10% SDS gels. The PCCT band was detected by chemiluminescence. The result is from a single experiment that is representative of results obtained from two different experiments.

molecular weight of 84 kD and in some cell lines (MDA-MB-468 and MDA-MB-231) the levels of this protein were greater than the levels of the 42 kD protein (Figure 34). Although the identity of this protein is not known, Dr. Kent's laboratory has informed us that the antibody recognizes this 84 kD protein in their cell extracts also and is likely to be a PCCT-related protein (personal communication).

Studies were performed to generate a calibration curve to define the range of cellular protein within which the PCCT levels detected with the antibody varied in a linear fashion. Increasing amounts of MCF-7 microsomal and cytosolic samples (2 - 20 μg protein) were separated on 10% SDS polyacrylamide gel. The proteins were transferred to nitrocellulose membranes which were then probed with the PCCT antibody. The PCCT band was detected by chemiluminescence (Figure 35A) and the images were scanned by a Fluor-SMax MultiImager and quantitated using the BioRad quantity one software (Figure 35B). The results revealed that the PCCT content varied linearly with up to 20 μg of microsomal protein and 15 μg of cytosolic protein (Figure 35B). Subsequent studies to determine the level of PCCT in microsomal fractions utilized quantities of protein within the linear range.

To examine whether the level of microsomal PCCT fluctuated in parallel to the enzyme activity in the cell cycle, 10 μg of solubilized microsomal proteins from synchronized cells at various stages of the cell cycle were separated on 10% SDS polyacrylamide gel. After transferring to nitrocellulose membrane, Western immunoblotting protocols with PCCT antibody were used to identify the band. The bands were quantitated by densitometric analysis. The results which are shown in Figure 36A and 36B demonstrated that PCCT levels in microsomal fractions isolated in late S and G2/M phase were 2-fold

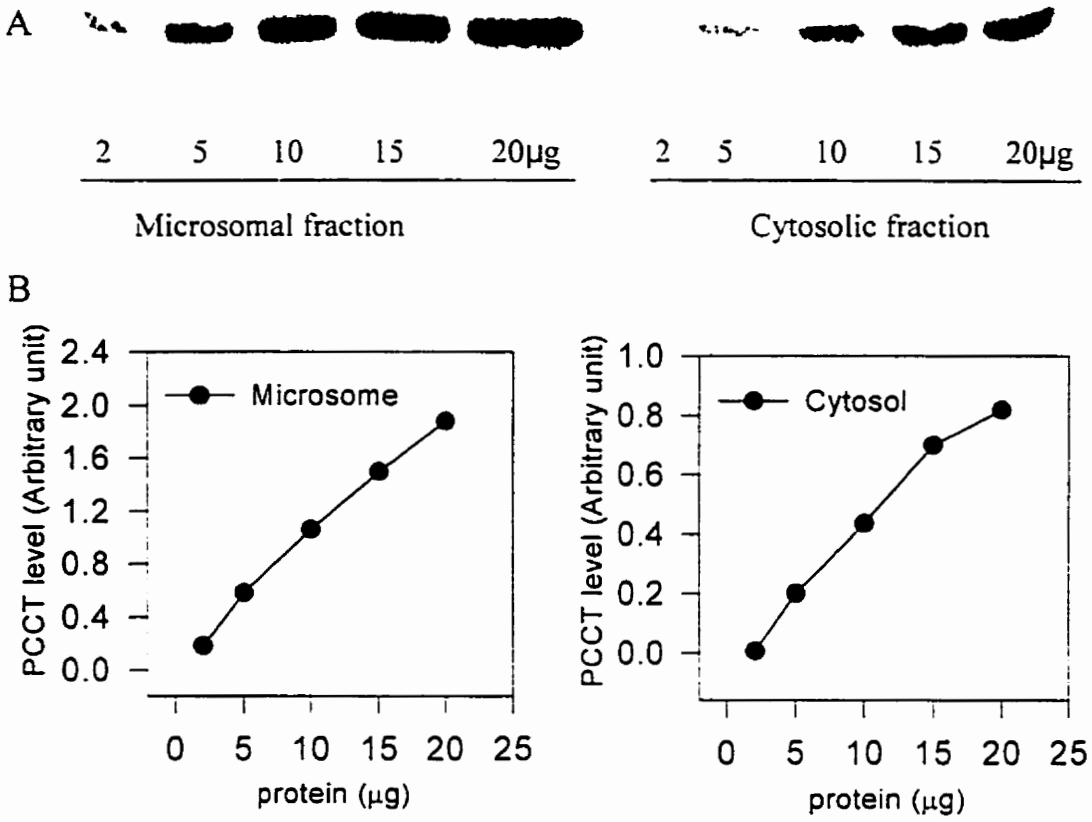


Figure 35. **Calibration curves for determination of PCCT levels in MCF-7 cells.** Known amounts of protein from both microsomal and cytosolic fractions of MCF-7 cells were separated by 10% SDS gel electrophoresis. Western immunoblotting procedures with PCCT antibody were used to identify the PCCT bands. Detection of the band was by chemiluminescence (A). The X-ray film was scanned with a Fluor-Smax MultiImager. PCCT levels were determined by densitometry analysis using BioRad quantity one software (B).

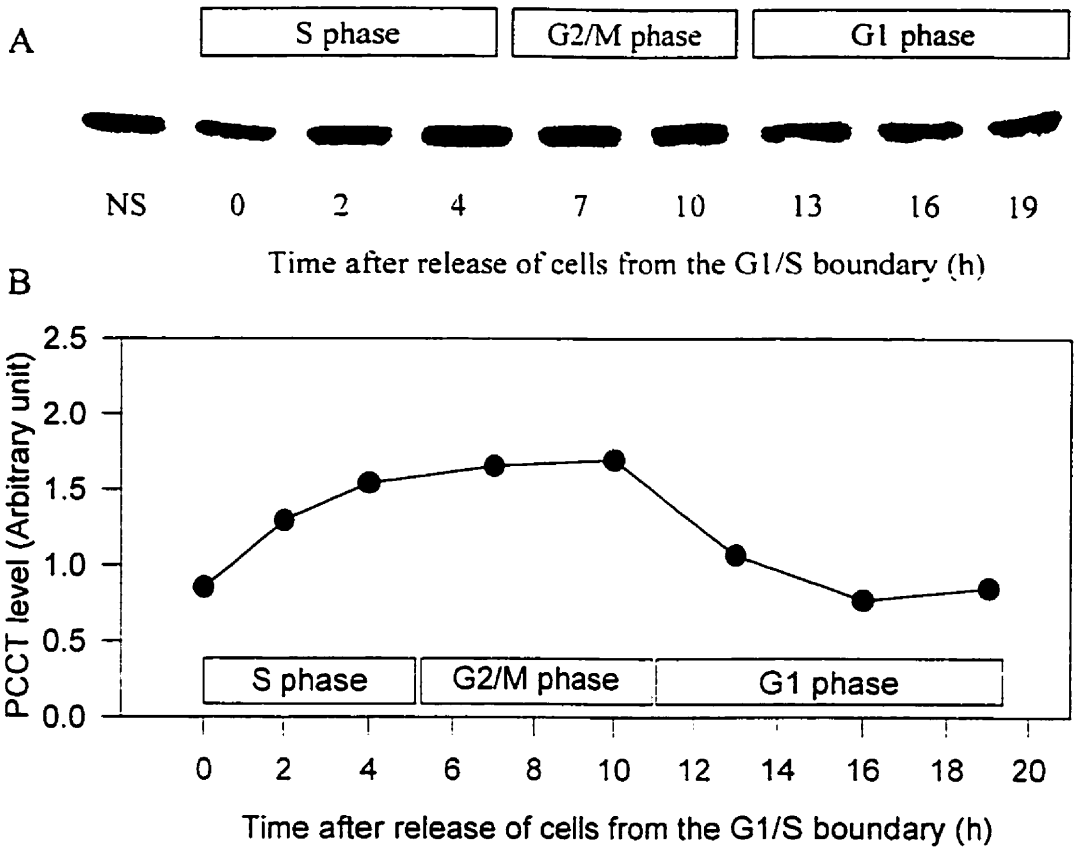


Figure 36. **Fluctuation of microsomal PCCT levels in the cell cycle.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary and subsequently incubated in 10% FBS-supplemented medium as described (section 2.2.2.1). The cells were harvested at selected intervals and subcellular fractions were prepared. 10 μg of solubilized microsomal proteins were separated on 10% SDS gels and transferred to nitrocellulose membranes for western blot analysis with PCCT antibody (A). The PCCT levels were quantified by densitometric analysis using BioRad quantity one software (B). NS: non synchronized cells.

greater than the levels in microsomes from cells at the G1/S boundary. As cells exited mitosis into G1 phase, the microsomal PCCT levels decreased to levels similar to those observed at the G1/S boundary (Figure 36). These changes in microsomal PCCT levels correlated somewhat with the observed fluctuation of PCCT activity (Figure 34).

3.4 CTP levels in the cell cycle

The similarity in changes to PC, PE, and PI content during the cell cycle observed in section 3.2 led to studies to investigate whether changes in cellular CTP levels could contribute to the cell cycle-related variation in the phospholipid content. The rationale for this was based on the following: at a constant rate of catabolism, the rate of biosynthesis regulates the rate of phospholipid accumulation. Because CTP is a common precursor required for the synthesis of all phospholipids, phospholipid synthesis in the cell cycle could be regulated by the fluctuation of CTP level during the cell cycle. A decrease in the CTP level could be caused by its conversion to dCTP for DNA synthesis during S phase which, if significant, could account for the observed decrease in phospholipid synthesis (Figure 28) and phospholipid content (Figure 25). Cessation of dCTP synthesis at the end of S phase would cause a rise in CTP levels and increased phospholipid synthesis (Figure 28).

To determine the CTP content in MCF-7 cells at different stages of the cell cycle, MCF-7 cells synchronized at the G1/S boundary were released from the blockage by removal of hydroxyurea. The cells were harvested at different times following the release and nucleotides were extracted and analyzed by HPLC as described in the experimental methods (section 2.2.19). The results showed that the CTP levels in the cells decreased by 20%

during early S phase (0 - 4 h). The level of CTP were restored as cell entered the G2/M phase and remained relatively constant for the remainder of the cell cycle (Figure 37). The results suggest that changes in CTP levels are unlikely to be primary regulators of phospholipid biosynthesis in the cell cycle, but as discussed later (section 4.3.2), more studies will be needed to draw firm conclusions on the role of CTP levels in regulating phospholipid synthesis in the cell cycle.

3.5 PC catabolism in the cell cycle

We have demonstrated that PC levels in MCF-7 cells decreases in the S phase of the cell cycle with an accumulation in the G2/M phase. As shown above, in MCF-7 cells, there is elevated [^{3}H]choline incorporation into PC during the G2/M phase (Figure 28) which correlated with an increase in the specific activity of PCCT (Figure 31) and an increase in microsomal PCCT levels (Figure 36). Together, these data suggest that elevated PC biosynthesis contributes to the observed PC accumulation in G2/M phase. Since the steady-state level of membrane phospholipid results from a balance between the rates of biosynthesis and degradation, studies were conducted to investigate the relative rate of PC catabolism at different stages of S and G2/M phases in order to assess whether changes in catabolism contribute to the accumulation of phospholipids in the cell cycle.

The approach utilized to study PC catabolism during the cell cycle involved labelling the cellular PC with trace amounts of [methyl-^{3}H]lysoPC during the synchronization procedures. Subsequently, the synchronized cells were chased in medium without the labelled precursor and hydroxyurea to allow the cells to progress along the cell cycle. The

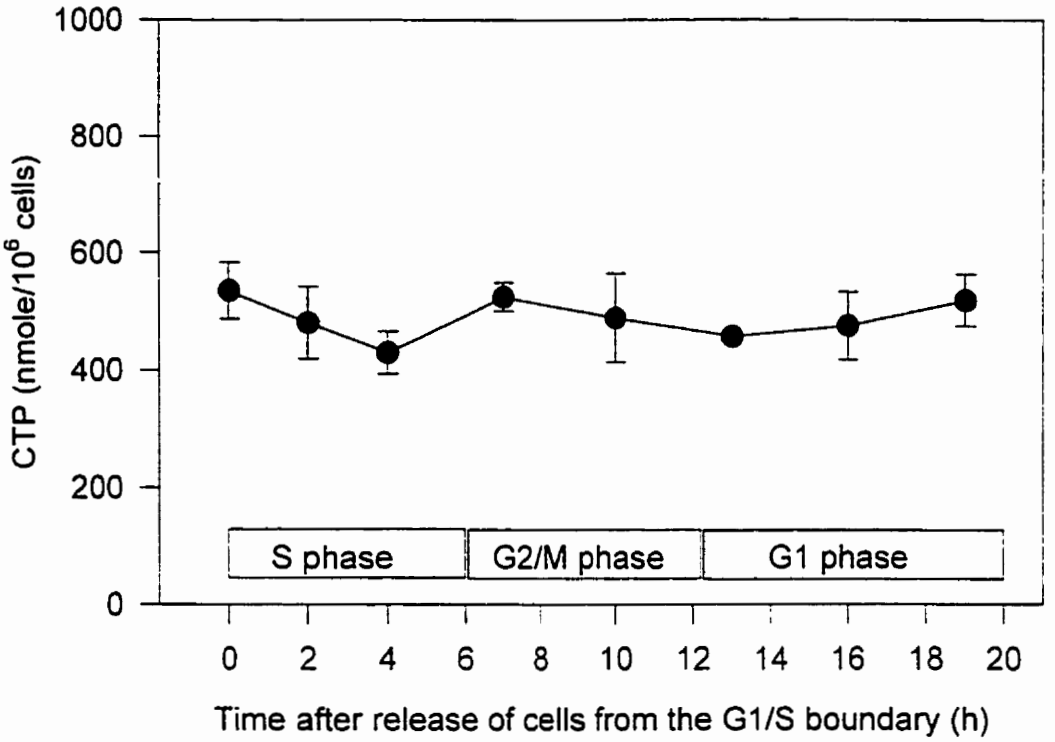


Figure 37. **CTP levels in the cell cycle.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary and subsequently incubated in 10% FBS-supplemented medium (section 2.2.2.1). The cells were harvested with trypsin at selected intervals. CTP was extracted from the cells as described in section 2.2.19. The CTP levels were determined by HPLC and expressed as nmole/10^6 cells. The results are the means ± S.D. of three independent experiments.

cells were harvested at selected times and the radioactivity in PC and water soluble choline metabolites were determined. Radioactivity in water soluble choline metabolites in the medium was also monitored in light of reports that the metabolites are rapidly excreted into the medium by cells (Morash *et.al.*, 1988 and 1989; Baburina and Jackowsk, 1999).

3.5.1 Optimum conditions for measuring PC catabolism

To study PC degradation during the cell cycle, studies were initially conducted to optimize the experimental conditions. We first established the incubation time with the [methyl-^{3}H]lysoPC that results in maximum labelling of PC in MCF-7 cells. Log-phase cells were labelled with [methyl-^{3}H]lysoPC for various times (0 - 10 h). At selected times, the medium was removed and the cells were harvested with trypsin. Lipids were extracted from the cells and separated by TLC as described in the experimental methods (section 2.2.5 and 2.2.6). Radioactivity incorporated in the phospholipids was determined by scintillation counting. The results demonstrated that more than 96% of the radioactivity in the phospholipids was associated with PC. LysoPC, SM, and PE only contained background radioactivity. The radioactivity incorporated in PC increased rapidly, peaked at 3 h and remained relatively constant thereafter (Figure 38). Incubation of the cells with [methyl-^{3}H]lysoPC for 3 h was therefore used to label PC in cells for all the subsequent studies.

After establishing the optimum labelling time, experiments were conducted to develop a protocol for the labelling and chase for the PC catabolism studies. Exponentially growing MCF-7 cells were synchronized at the G1/S boundary by the double hydroxyurea treatment (section 2.2.2.1). The medium was replaced with one containing [methyl-

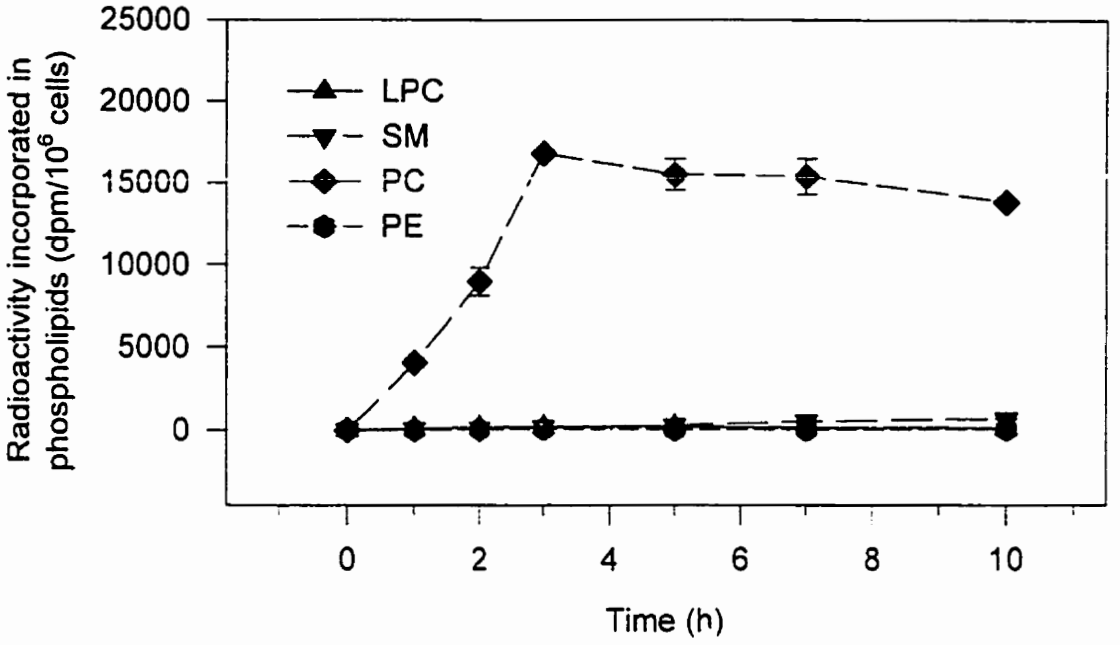


Figure 38. **Incorporation of [methyl-^{3}H]lysoPC into phospholipids.** Exponentially growing MCF-7 cells were incubated with [methyl-^{3}H]LPC (1.5 x 10^6 dpm/dish). At selected intervals, the cells were washed three times with Hank's solution and harvested with trypsin. Lipids were extracted as described in section 2.2.5. The phospholipids were separated into individual components by TLC with chloroform/methanol/HOAc/H_2O (70:30:2:4, by vol). The radioactivity in each fraction was determined by scintillation counting. The results are the means ± S.E. of two separate experiments with duplicated determination.

^{3}H]lysoPC and hydroxyurea during the last 3 h of incubation with hydroxyurea. Subsequently, the label medium was removed, the cells were washed three times with Hank's solution and incubated in 10% FBS supplemented-medium to allow cell progression along the cell cycle. At hourly intervals for up to 12 h, the cells were washed three times with Hank's solution and harvested with trypsin. Lipids were extracted from both the medium and the cells (section 2.2.5). The phospholipid classes in the organic phase of the cell extract were separated by TLC (section 2.2.6). The radioactivity associated with PC was determined by scintillation counting. The results showed that radioactivity associated with PC remained relatively constant during early S phase (0 - 2 h of the chase), and significantly decreased in mid- to late S phase (between 2 - 6 h of the chase) by approximately 50% and remained relative constant during the G2/M phase (Figure 39).

The results from the analysis of the choline metabolites in the aqueous phase of both the medium and the cell extracts (Figure 39) revealed that increasing amounts of radioactivity was associated with GPC and P-Cho in S and G2/M phases. After 3 h of the chase, the combined levels of radioactivity associated with GPC and P-Cho in both the cell and the medium were higher than the levels in cellular PC at the start of the chase (Figure 39). This suggests that in addition to production of these metabolites from PC, there was probably contribution from residual [^{3}H-methyl]lysoPC during the chase. In order to avoid this, the cells were washed with 1% BSA containing PBS to remove lysoPC (Morash *et al.*, 1988) and incubated for an additional 2 h in the absence of [methyl-^{3}H]lysoPC following cell labelling to empty the [methyl-^{3}H]lysoPC pool. In addition, the medium was replaced once to remove choline containing metabolites in the medium during the 2 h incubation which

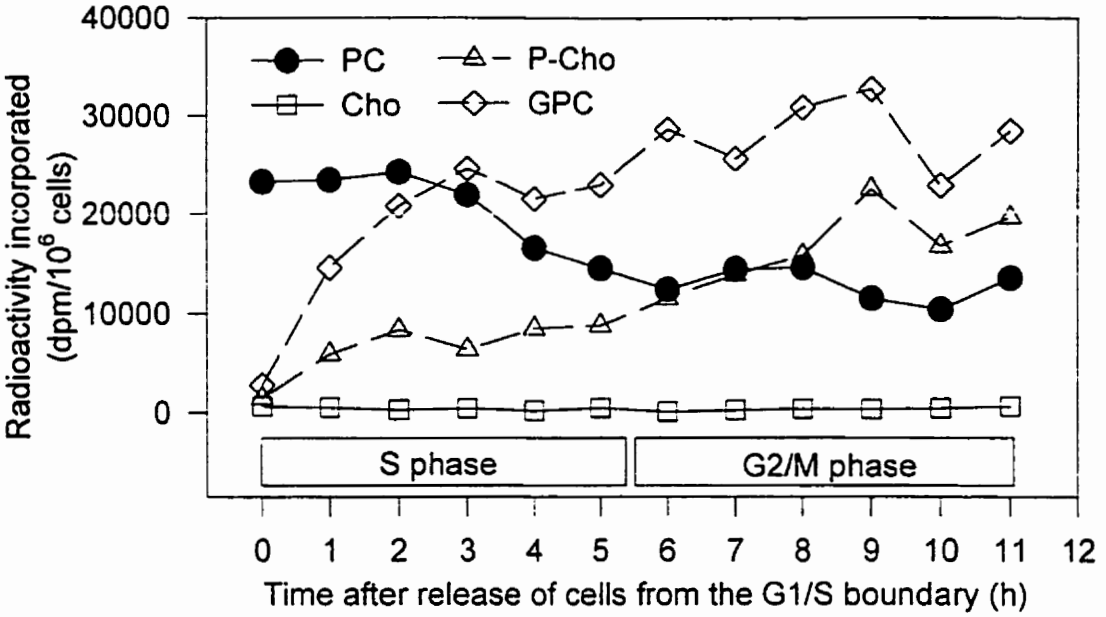


Figure 39. **PC turnover during S and G2/M phases.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary as described in section 2.2.2.1. During the last 3 h of cell synchronization, the cells were incubated with [^{3}H-methyl]lysoPC. Subsequently, the cells were incubated in 10% FBS-supplemented medium without hydroxyurea and [^{3}H-methyl]lysoPC. At the selected intervals, the cell were harvested with trypsin. Lipids were extracted from both the medium and the cells (section 2.2.5). Phospholipids and choline-containing metabolites were separated by TLC as described. The radioactivity in individual fractions was determined by scintillation counting. The radioactivity associated with choline-containing metabolites are the sum of the radioactivity recovered from both the medium and the cells.

would reduce the recycling of the label choline metabolites back into PC.

3.5.2 PC catabolism in exponentially growing MCF-7 cell

The protocol established above for the studies of PC catabolism was first examined in exponentially growing MCF-7 cell. MCF-7 cells were labelled in medium containing [methyl-^{3}H]lysoPC for 3 h. The labelled medium was removed and the cells were washed three times with PBS+1% BSA. Subsequently, the cells were incubated in 10% FBS-supplemented medium for 2 h, during which the medium was replaced once. The cells were then washed three times with PBS+1% BSA and incubated in 10% FBS-supplemented medium. At 2 h intervals between 0 - 12 h, the medium was removed and the cells were detached with trypsin, the cells were subjected to a biphasic extraction (section 2.2.5). Phospholipids and the choline-containing metabolites were separated (section 2.2.6 and 2.2.7) by TLC and the radioactivity associated with PC and choline-containing metabolites (section 3.6.2) was determined. The results showed that during the 12 h chase, there was a linear decrease in the radioactivity associated with cellular PC with a $t_{\frac{1}{2}}$ of approximately 10 h (Figure 40). Very little radioactivity was associated with lysoPC, SM, or PE and these did not change significantly during the chase (Figure 40). Similar results were obtained when the cells were labelled with [1-palmitoyl-^{14}C]lysoPC except that in the later experiment the PE fraction was significantly labelled (Figure 41). Therefore, labelling cells for 3 h followed by an additional 2 h incubation in the absence of radiolabel precursor before chasing was used for all subsequent experiments to study PC catabolism in the cell cycle.

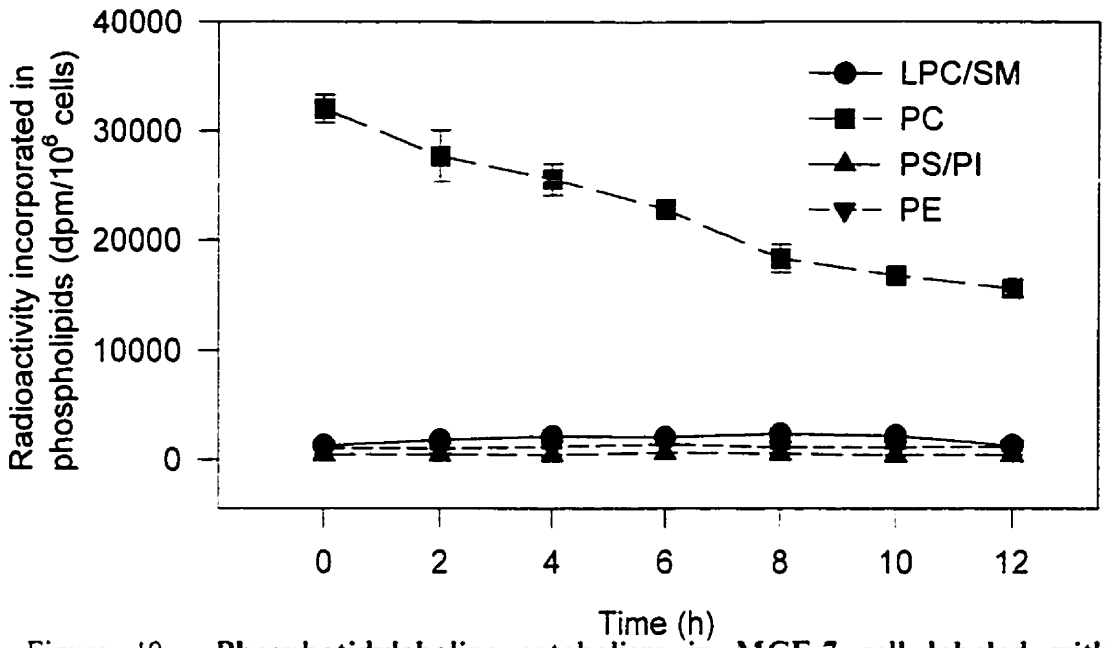


Figure 40. **Phosphatidylcholine catabolism in MCF-7 cell labeled with [methyl-^{3}H-]lysoPC.** Exponentially growing MCF-7 cells were incubated with [methyl-^{3}H]lysoPC for 3h. The medium was removed and the cells were incubated in the absence of labelled precursor for 2 h to empty the [methyl-^{3}H]lysoPC residue. Subsequently, the cells were incubated in 10% FBS-supplemented medium. At the selected intervals, duplicated dishes of cells were harvested with trypsin. Lipids were extracted and separated by TLC as described in the experimental section. Radioactivity associated with individual phospholipids was determined by scintillation counting. The results are the means ± S.E. of two separate experiments analyzed in duplicate.

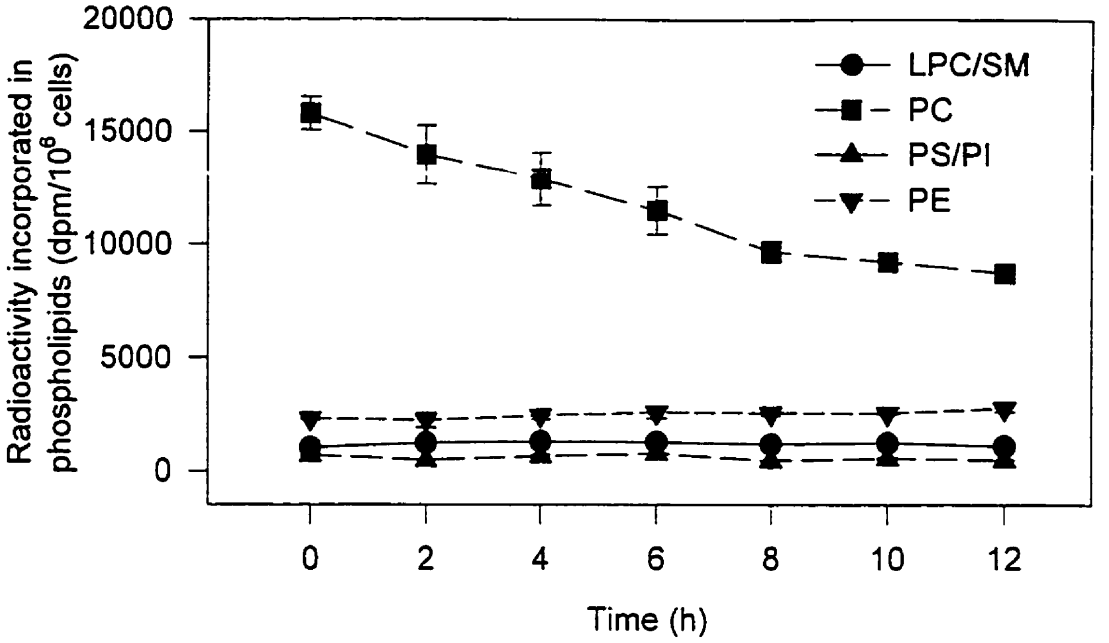


Figure 41. **Phosphatidylcholine catabolism in MCF-7 cell labeled with [1-palmitoyl-^{14}C]lysoPC.** Exponentially growing MCF-7 cells were incubated with [1-palmitoyl-^{14}C]lysoPC for 3h. The medium was removed and the cells were incubated in the absence of the labelled precursor for 2 h. and subsequently with 10% FBS-supplemented medium. At the selected intervals, the cells were harvested with trypsin and lipids were extracted and separated by TLC as described in section 2.2.5 and 2.2.6. Radioactivity associated with individual phospholipids was determined by scintillation counting. The results are the means ± S.E. of two separate experiments analyzed in duplicate.

3.5.3 PC catabolism during S and G2/M phases

To investigate changes in PC catabolism during S and G2/M phases, exponentially growing MCF-7 cells in 60 mm dishes were synchronized at the G1/S boundary by the double hydroxyurea treatment as previously described (section 2.2.2.1). The cells were labelled with [methyl-^{3}H]lysoPC for 3 h during the last stages of the synchronization procedure (section 2.2.16). Subsequently, the radiolabelled medium was removed and the cells were incubated in complete growth medium with hydroxyurea but without labelled precursor for an additional 2 h. The cells were incubated in 10% FBS supplemented medium to allow cell progression along the cell cycle and the radioactivity associated with cellular PC was chased to study PC catabolism during S and G2/M phase. The loss of radioactivity associated with PC was monitored as an indicator of PC degradation and the results are displayed in Figure 42. The results showed that the level of radioactivity associated with PC decreased approximately 23% 4 h after release of the cells from the G1/S boundary and remained relatively constant from mid- to late S phase and throughout the G2/M phase (Figure 42). These results are an indication that there was accelerated degradation of PC in early to mid S phase which was attenuated in the G2/M phase.

To confirm the cessation of PC degradation in the G2/M phase. Double hydroxyurea block synchronized cells were released from the G1/S boundary by washing the cells three times with Hanks' solution. The cells were incubated in 10% FBS-supplemented medium to allow cell progression along the cell cycle. After 1 h incubation, the medium was replaced with one containing [methyl-^{3}H]lysoPC and the cells were labelled for 3 h. The labelled medium was removed and the cells were incubated in 10% FBS-supplemented

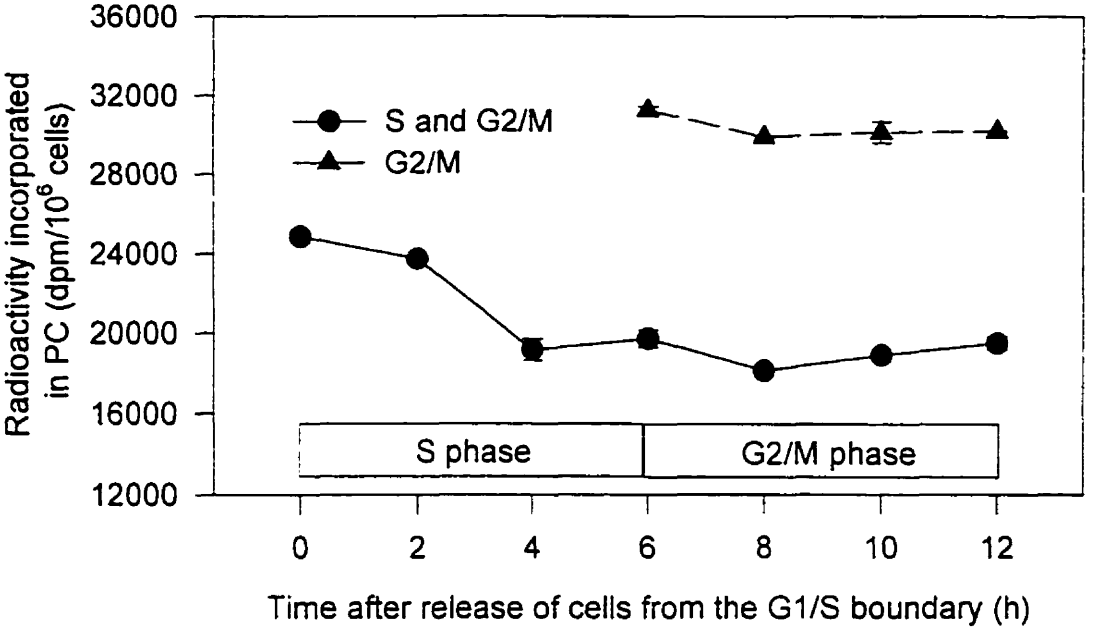


Figure 42. **PC catabolism during S and G2/M phases.** Exponentially growing MCF-7 cells were synchronized at the G1/S boundary, labeled with [^{3}H-methyl]lysoPC for 3 h and subsequently incubated in 10%-FBS supplemented medium without [^{3}H-methyl]lysoPC for 2 h before they were released from the G1/S boundary (section 2.2.16). To confirm PC turnover during the G2/M phase, Synchronized cells released from the G1/S boundary were pulse labeled with [^{3}H-methyl]lysoPC for 3 h during S phase before they were chased in G2/M phase (section 2.2.16). At the selected intervals, the cell were harvested with trypsin. Lipids were extracted and separated by TLC. Radioactivity associated with PC were determined by scintillation counting. The results are the means ± S.E of two separate experiments analyzed in duplicate.

medium for an additional 2 h (between 4 ~ 6 h post the G1/S boundary) during which the medium was replaced once. The cells were then incubated in 10% FBS-supplemented medium for 6 h. At 2 h intervals corresponding to 6, 8, 10, 12 h after release from the G1/S boundary, the cells were harvested with trypsin. Lipids were extracted from the cells and separated by TLC (section 2.2.6). Radioactivity associated with PC was determined and expressed as dpm/10^6 cells. The results demonstrated that the level of radioactivity associated with PC was unchanged during the G2/M phase (Figure 42), confirming the results obtained in studies where the cells were labelled during the synchronization procedures. These results are consistent with previous observation that there was minimal PC degradation during the G2/M phase of the cell cycle in BAC1.2F5 cells (Jackowski, 1994).

3.6 Generation of GPC in MCF-7 cells

As discussed in the previous section (section 3.5.1), labelling of MCF-7 cells with [methyl-^{3}H]lysoPC led to a rapid incorporation of radioactivity into PC with a very little radioactivity found in cellular lysoPC, SM, PS, PI and PE (Figure 38). When the loss of radioactivity in the medium lysoPC and the appearance of label in choline metabolites in the medium and the cell were analyzed, we observed that the majority of the label that was progressively lost from lysoPC was recovered in the medium as GPC (Figure 43). Other compounds that were significantly labelled were cellular PC and medium Cho. Medium P-Cho and cellular Cho fractions contained only background radioactivity (data not shown). The observation that there was much more rapid and greater accumulation of the label in the

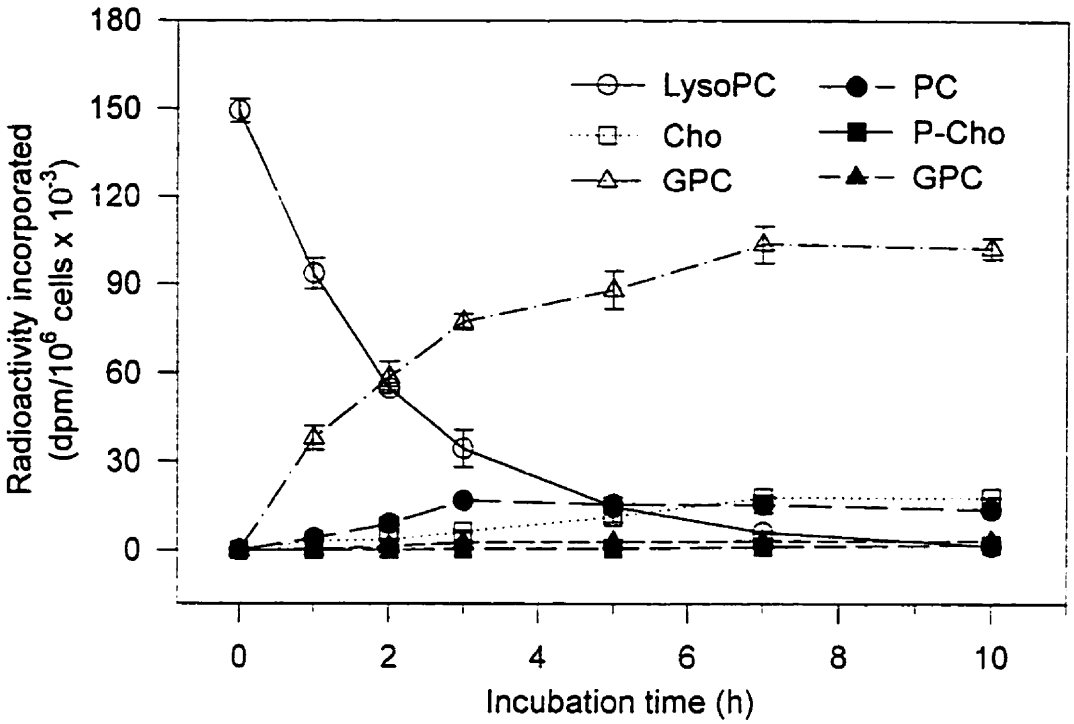


Figure 43. **Metabolism of exogenous [methyl-^{3}H]lysoPC in MCF-7 cells.** Exponentially growing MCF-7 cells were incubated with [methyl-^{3}H]LPC (1.5 x 10^6 dpm/dish). At selected intervals, the medium was collected and the cells were harvested with trypsin. Lipids were extracted from both the medium and cells as described in section 2.2.5. The phospholipids and choline catabolite were separated by TLC (section 2.2.6). The radioactivity in each fraction was determined by scintillation counting. The results are the means ± S.E. of two separate experiments analyzed in duplicate. The open and closed symbols represent medium and cellular fractions respectively.

medium GPC than the cellular PC suggested that the medium GPC was being generated from sources other than via PC hydrolysis. Clearly, non-enzymatic breakdown of lysoPC in the medium could account for these observation especially since high levels of GPC and Cho were found in the medium. The stability of lysoPC in 10% FBS-supplemented medium was therefore investigated. [methyl-^{3}H]lysoPC was incubated in FBS-supplemented medium for 10 h in the absence of cells. Analysis of the radioactivity in lysoPC and choline containing metabolites revealed 97.9% of the radioactivity was associated with lysoPC with only very small amount of radioactivity present in Cho, P-Cho, and GPC fractions. This result indicates that [methyl-^{3}H]lysoPC is chemically stable. Hence, the high levels of medium GPC and Cho observed were produced as a consequence of cellular enzymatic activity.

GPC has long been recognized as the major catabolite of PC (Morash *et al.*, 1988 and 1989; Walkey, 1994), and a large accumulation of [^{3}H]GPC was observed in the culture medium when cells were incubated with [methyl-^{3}H]lysoPC (Morash *et al.*, 1988 and 1989; Baburina and Jackowski, 1999). Current dogma suggests that the medium GPC is secreted from the cytosol following degradation of PC in the cell (Morash *et al.*, 1988 and 1989; Baburina and Jackowski, 1999). However, the high levels of the medium GPC relative to the low levels of cellular PC and GPC (Figure 43) suggests that the medium GPC was unlikely to be derived from secretion of cellular GPC produced by degradation of PC. It seemed more likely that direct deacylation of medium lysoPC in the outer lipid bilayer by lysophospholipase was the probably cause of the GPC accumulation in the medium.

3.6.1 Generation of GPC in the presence of exogenous labelled lysoPC

To determine whether medium GPC was derived directly from deacylation of lysoPC by lysophospholipase or secreted from the cells, exponentially growing cells were incubated in medium containing both [methyl-^{3}H]lysoPC and [1-palmitoyl-^{14}C]lysoPC for up to 3 h. The rationale was that if lysoPC was directly degraded by lysophospholipases, there should be a rapid accumulation of [^{3}H]GPC as well as [^{14}C]free fatty acid (FA) in the medium. On the other hand, if lysoPC was first acylated to PC, degraded and subsequently secreted into medium, we should observe a rapid and large accumulation of [^{3}H]choline-containing metabolites (Cho, P-Cho, and GPC), as well as [^{14}C]palmitoyl-containing metabolites (FA, MAG, DAG, TAG, and FA-CoA) in the cell.

Exponentially growing cells were incubated in medium with [methyl-^{3}H]lysoPC and [1-palmitoyl-^{14}C]lysoPC for 3 h. At 30 min intervals, the cells were harvested and lipids were extracted from both the medium and the cells and separated by TLC. Radioactivity associated with different fractions were determined by scintillation counting. The data are expressed as a percentage of total radioactivity recovered from the medium and the cells (Table 9 and 10). When MCF-7 cells were incubated with [methyl-^{3}H]lysoPC, the levels of medium lysoPC decreased rapidly from 100% to 8.5% within 180 min incubation (Table 9). After 3 h incubation, 3.8%, 3.0% and 70.2% of the radioactivity were recovered in medium Cho, P-Cho and GPC, respectively, while radioactivity associated with PC accounted for only 12.5%. Only background radioactivity was found in other phospholipids (data not shown). Very little radioactivity was associated with cellular Cho, P-Cho and GPC (Table 9). The faster and greater accumulation of the radioactivity in the medium GPC rather than

Table 9. **Distribution of radioactivity in MCF-7 cells incubated with [methyl-^{3}H]lysoPC.** MCF-7 cells were incubated with [methyl-^{3}H]lysoPC. At the selected intervals, the medium was removed and the cells harvested by trypsin. Lipids were extracted from both the medium and the cells (section 2.2.5). The phospholipids and choline-containing metabolites were separated by TLC (section 2.2.6 and 2.2.7). Radioactivity incorporated in different components were determined by scintillation counting. Results were expressed as a percentage of the total radioactivity recovered from both the medium and the cells. The results are the means ±S.E. of two separate experiments analyzed in duplicate.

Distribution of radioactivity (% of total radioactivity recovered in both the medium and the cells)								
	Medium					Cell		
Time (min)	LysoPC	Cho	P-Cho	GPC	PC	Cho	P-Cho	GPC
0	100	-	-	-	-	-	-	-
30	53.1 ± 3.3	1.1 ± 0.8	1.3 ± 0.6	36.7 ± 2.0	7.3 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	0.5 ± 0.0
60	31.7 ± 1.2	1.5 ± 0.6	3.9 ± 0.4	52.7 ± 3.0	9.4 ± 1.7	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2
90	21.2 ± 2.8	1.9 ± 0.4	4.0 ± 1.4	60.8 ± 5.9	10.9 ± 0.2	0.1 ± 0.0	0.2 ± 0.0	1.0 ± 0.1
120	15.2 ± 0.9	2.1 ± 0.1	2.0 ± 1.5	67.9 ± 2.0	11.4 ± 0.4	0.1 ± 0.0	0.2 ± 0.0	1.1 ± 0.1
150	10.4 ± 0.5	2.8 ± 0.2	3.4 ± 2.0	69.3 ± 2.1	12.4 ± 0.5	0.0 ± 0.0	0.3 ± 0.0	1.4 ± 0.1
180	8.5 ± 0.5	3.8 ± 0.5	3.0 ± 1.6	70.2 ± 0.5	12.5 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	1.6 ± 0.1

the cellular PC coupled with the lack of accumulation of cellular choline-containing metabolites suggested that the majority of the medium GPC was not secreted from the cells but derived from direct deacylation of medium lysoPC.

During the 3 h incubation of the cells with [1-palmitoyl-^{14}C]lysoPC, the levels of the medium [1-palmitoyl-^{14}C]lysoPC decreased rapidly from 100% to 14.4% (Table 10). Of the total radioactivity recovered in the cells and the medium after 3 h incubation, 44.8% of the radioactivity was recovered in the medium as free fatty acid (FA), while the radioactivity associated with PC and PE accounted for 33.6% and 3.2% respectively (Table 10). Little radioactivity was observed in cellular palmitoyl-containing catabolites (FA, MAG, DAG, TAG, and FA-CoA) (Table 10). Incubation of [1-palmitoyl-^{14}C]lysoPC in the absence of cells revealed that 98.6% of the radioactivity was recovered from lysoPC. This again demonstrated that lysoPC is chemically stable. The more rapid and greater accumulation of the labelled FA in the medium rather than the cellular PC clearly demonstrated that majority of lysoPC was hydrolyzed by cellular lysophospholipases. The lack of accumulation of radiolabelled palmitic acid in cellular metabolites also supports the ideal that the medium GPC and FA are not secreted from the cell.

Taken together, our results clearly demonstrated that when lysoPC was present in the medium, the majority of the medium GPC was derived from direct hydrolysis of lysoPC by lysophospholipase rather than secretion from the cells.

3.6.2 Generation of GPC in the absence of exogenous labelled lysoPC

In the previous section, we demonstrated that in the presence of exogenous labelled

Table 10. **Distribution of radioactivity in MCF-7 cells incubated with [1-palmitoyl-^{14}C]lysoPC.** MCF-7 cells were incubated with [1-palmitoyl-^{14}C]lysoPC. At the selected intervals, the medium was removed and the cells harvested by trypsin. Lipids were extracted from both the medium and the cells (section 2.2.5). Phospholipids and choline-containing metabolites were separated by TLC (section 2.2.6 and 2.2.7). Radioactivity incorporated in different components were determined by scintillation counting. Results were expressed as a percentage of the total radioactivity recovered from both the medium and the cells. The results are the means ±S.E. of two separate experiments analyzed in duplicate.

Distribution of radioactivity (% of total radioactivity recovered in both the medium and the cells)									
Time	Medium		Cellular phospholipids		Cellular palmitoyl-containing catabolites				
(min)	LysoPC	FA	PC	PE	FA	MG	DG	TG	upper phase
0	100	-	-	-	-	-	-	-	-
30	66.8 ± 3.5	18.3 ± 2.7	12.7 ± 0.8	0.8 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.0
60	45.3 ± 0.8	33.0 ± 2.3	18.4 ± 2.4	1.2 ± 0.2	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
90	31.3 ± 2.7	37.3 ± 4.2	26.2 ± 1.6	2.3 ± 0.2	0.4 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	0.7 ± 0.0
120	24.3 ± 0.2	42.0 ± 2.1	28.7 ± 2.3	2.3 ± 0.2	0.4 ± 0.0	0.6 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	0.8 ± 0.1
150	17.1 ± 0.5	44.8 ± 3.9	31.4 ± 3.5	2.9 ± 0.4	0.6 ± 0.0	0.7 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	0.9 ± 0.2
180	14.4 ± 1.4	44.8 ± 2.0	33.6 ± 2.6	3.2 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.0	1.2 ± 0.2	0.9 ± 0.1

lysoPC, the majority of the medium GPC was derived from direct hydrolysis of exogenous labelled lysoPC by lysophospholipases. We next investigated whether in the absence of medium lysoPC, such as the conditions we utilized in the pulse-chase studies to study PC catabolism (section 3.5.2), significant levels of GPC were found in the medium, in view of its reported secretion by cells (Morash *et al.*, 1988 and 1989; Baburina and Jackowski, 1999). Exponentially growing cells were pulse labelled with [methyl-^{3}H]lysoPC for 3 h to label PC in cells. Subsequently, the medium was removed and the cells were incubated for 2 h in the absence of labelled precursor. The cells were then chased with 10% FBS-supplemented medium and harvested at every 2 h intervals for up to 12 h. Lipids were extracted from both the cells and the medium and separated by TLC. Radioactivity associated with different fractions were determined by scintillation counting. Of the total radioactivity recovered from both the medium and the cell at time 0, 87.2% and 7.6% of the radioactivity were associated with PC and intracellular GPC respectively (Table 11). During the 12 h chase, the label in PC decreased from 87.2% to 39.1%. Analysis of the aqueous phase of the cell extract revealed that the levels of cellular P-Cho and GPC increased from 2.0% to 4.8% and 7.6% to 11.0% respectively, while cellular Cho fraction had only background radioactivity during the chase (Table 11). In the medium, radioactivity in Cho increased from 5.9 to 19.6%, while in GPC the increase was from 11.7% to 20.1%. These results showed that even in the absence of medium [methyl-^{3}H]lysoPC, a significant amount of radioactivity was recovered in the medium GPC and Cho.

Studies were conducted to investigate whether medium GPC was generated by sequential deacylation of PC in the outer lipid bilayer of the cells or generated inside the cells

Table 11. **Distribution of radioactivity in different fractions after pulse-chase with [methyl-^{3}H]lysoPC.** Exponentially growing MCF-7 cells were pulsed labelled with [methyl-^{3}H]lysoPC for 3 h and subsequently incubated in the absence of labelled precursor for additional 2 h before they were chased in 10% FBS-supplemented medium for 12 h. The cells were harvested with trypsin. Lipids were extracted from both the cells and the medium. The radioactivity associated in phospholipids as well as choline containing catabolites were determined. Results were expressed as relative percentages of the total radioactivity recovered in both the medium and the cells. The results are means ± S.E. of two separate experiments analyzed in duplicate.

Distribution of radioactivity (% of total radioactivity recovered in both the medium and the cells)								
	Phospholipids		Cellular choline-containing catabolites			Medium choline-containing catabolites		
Time (h)	PC	PE	Cho	P-Cho	GPC	Cho	P-Cho	GPC
0	87.2 ± 1.2	2.8 ± 0.2	0.5 ± 0.2	2.0 ± 0.6	7.6 ± 0.2	-	-	-
2	63.7 ± 0.9	2.5 ± 0.8	0.5 ± 0.3	2.8 ± 0.1	11.0 ± 0.8	5.9 ± 0.4	2.0 ± 0.1	11.7 ± 1.2
4	55.4 ± 1.8	2.6 ± 0.6	0.5 ± 0.4	4.0 ± 0.5	11.4 ± 0.3	9.7 ± 0.4	4.3 ± 1.1	12.1 ± 2.7
6	49.9 ± 2.4	3.0 ± 0.5	0.7 ± 0.1	3.6 ± 0.5	12.0 ± 0.7	11.2 ± 0.8	1.9 ± 0.5	11.9 ± 2.7
8	44.9 ±0.5	2.7 ± 0.3	0.3 ± 0.2	3.9 ± 0.6	10.9 ± 1.3	18.0 ± 2.8	3.4 ± 1.3	15.9 ± 37
10	39.3 ± 1.0	2.6 ± 0.5	0.5 ± 0.1	3.7 ± 0.1	9.6 ± 0.2	19.4 ± 1.4	1.9 ± 1.1	22.9 ± 3.7
12	39.1 ± 1.1	2.9 ± 0.1	0.7 ± 0.1	4.8 ± 0.5	11.0 ± 1.8	19.6 ± 1.5	1.9 ± 0.4	20.1 ± 2.4

and subsequently secreted into the medium. The above experiment was repeated with [1-palmitoyl-^{14}C]lysoPC. The rational was that if GPC is generated from PC in the outer lipid bilayer by the action of PLA and lysoPLA, we should observe an accumulation of labelled fatty acid in the medium, otherwise, we should observe an elevation of labelled fatty acid in the cells but not in the medium because fatty acids are not secreted from the cell into the medium. Our results showed that after 2 h of the chase, the levels of label in PC decreased from 82.1% to 74% but the labelling in cellular FA decreased from 1.6% to 1.2% (Table 12). On the other hand, the label in medium FA increased to 8.4% (Table 12). With increasing duration of the chase, the proportion of label in PC continued to decrease to about 62%, while the amount of label in medium FA increased to 15% at 8 h followed by decreased to 12% 12 h after the chase (Table 12). Very little changes were observed in the proportion of the label in the cellular FA and any other potential palmitoyl-containing metabolites (MAG, DAG, TAG, and FA-CoA) with time. The proportion of labelled PE was 12.0% at the beginning of the chase and remained relative unchanged during the early chase (0 ~ 6 h) but progressively increased to approximately 20.0% at 12 h (Table 12). The lack of accumulation of palmitoyl in cellular metabolites coupled with the accumulation of labelled FA in the medium suggest that in the absence of exogenous labelled lysoPC, medium labelled metabolites resulted from hydrolysis of the labelled PC in the outer leaflet of the plasma membrane by PLA and lysoPLA rather than generation inside the cell and subsequent secretion into the medium. The elevation of label in PE during late chase is probably due to recycling of the labelled FA released in the medium. The observation of a slight decrease of medium FA during late chase supports the above hypothesis. Taken together, the above

Table 12. **Distribution of radioactivity in different fractions after pulse-chase with [1-palmitoyl-^{14}C]lysoPC.** Exponentially growing MCF-7 cells were pulsed labelled with [1-palmitoyl-^{14}C]lysoPC for 3 h and subsequently incubated in the absence of labelled precursor for additional 2 h before they were chased in 10% FBS-supplemented medium for 12 h. The cells were harvested with trypsin and lipids were extracted from both the cells and the medium. Radioactivity associated with the phospholipids as well as palmitoyl-containing catabolites were determined. Results were expressed as relative percentages of the total radioactivity recovered in both the cells and the medium. The results are the means ± S.E. of two separate experiments analyzed in duplicate.

Distribution of radioactivity (% of total radioactivity recovered in both the medium and the cells)								
	Phospholipids		Cellular palmitoyl-containing catabolites					Medium
Time (h)	PC	PE	FA	MG	DG	TG	upper phase	FA
0	82.1 ± 0.0	12.0 ± 0.5	1.6 ± 0.1	1.1 ± 0.1	1.9 ± 0.2	0.3 ± 0.2	1.1 ± 0.4	-
2	74.0 ± 0.9	13.0 ± 0.9	1.2 ± 0.2	0.7 ± 0.1	1.3 ± 0.2	0.4 ± 0.0	1.1 ± 0.0	8.4 ± 0.1
4	67.9 ± 2.5	12.9 ± 0.0	1.2 ± 0.4	0.9 ± 0.0	1.6 ± 0.2	0.4 ± 0.1	1.1 ± 0.1	14.0 ± 2.6
6	65.8 ± 1.8	13.5 ± 0.8	1.5 ± 0.3	0.7 ± 0.3	1.6 ± 0.2	0.4 ± 0.1	1.1 ± 0.1	15.4 ± 2.6
8	63.7 ± 0.2	16.5 ± 0.3	1.3 ± 0.4	1.0 ± 0.2	1.6 ± 0.3	0.6 ± 0.2	1.0 ± 0.1	14.8 ± 1.1
10	63.2 ± 0.7	17.5 ± 0.1	1.4 ± 0.2	1.1 ± 0.1	1.4 ± 0.0	0.4 ± 0.2	0.7 ± 0.2	14.0 ± 0.3
12	62.4 ± 0.1	19.6 ± 0.9	1.4 ± 0.3	1.0 ± 0.2	1.4 ± 0.3	0.4 ± 0.0	0.9 ± 0.1	12.8 ± 0.8

results indicate that the medium GPC was generated by sequential deacylation of PC in the outer leaflet of the plasma membrane rather than hydrolysis of PC to GPC inside the cells and subsequent secretion of the GPC into the medium.

4 DISCUSSION

Cells reproduce by duplicating their components (chromosomes, organelles, and biological membranes), doubling their size and dividing in two. Since phospholipids are major components of cell membranes, cells must double their phospholipid mass so that the daughter cells formed have the same membrane composition as the parent. Because discordant regulation of phospholipid accumulation by only a few percent per cell cycle would rapidly result in cells with either a large excess or deficit of membrane surface leading to abnormalities in cell size and/or intracellular lipid accumulation (Jackowski, 1994 and 1996), therefore, stringent control mechanisms to keep the phospholipid content in turn with the cell cycle are expected. However, how membrane phospholipid accumulation is regulated within the cell cycle remains largely unknown (Jackowski, 1996). We have therefore investigated the coordination and regulation of phospholipid accumulation in the cell cycle.

4.1 Cell model and cell synchronization

The human mammary adenocarcinoma cell line, MCF-7, was selected as the cell model for the project after initial studies with human normal epidermal keratinocytes and human normal mammary epithelial cells revealed that the limited life span of the normal cells made their use impractical for generating the large numbers of cells we required for our studies. In addition, the levels of synchronization obtained with these cells were not as high

as we desired.

The large number of cells required to study the regulation of phospholipid production in the cell cycle were obtained by synchronizing the cells with the chemical agents, hydroxyurea and nocodazole. Generally, synchronization of cells can be achieved by arresting cell progression at a particular point in the cell cycle with drugs or by physically isolating subpopulations of cells at different phases of the cell cycle by exploiting cellular properties that change as cells go through the cell cycle. Two commonly used selection procedures are centrifugal elutriation which sorts the cells by size and fluorescence-activating cell sorting which separates the cells by their DNA content. Although selection synchronization procedures avoid the use of chemical agents that might perturb the normal biochemical processes of the cell, they generate only small quantities of the cells at different phases of the cell cycle. The population of cells obtained from centrifugal elutriation also tend to contain overlapping cells at different stages of the cell cycle (Krek and Decaprio, 1995; Bosc, 1999), while fluorescence-activating cell sorting can only sort cells into three classes (G1, S, and G2/M phase). In addition, synchronized progression is difficult to achieve with cells obtained by these selection procedures because even cells from a single phase include those that have just entered the phase right through to those that are about to exit the phase. For the above reasons, selection synchronization procedures were not suitable for our study. Two synchronization procedures were developed because the existing induction synchronization procedure did not yield the high degree of synchronization we sought, or they failed to generate highly synchronized cell movement after synchronized cells were released (data not shown). Previous studies in our lab revealed that the doubling time

of MCF-7 cells is approximately 20 h (Lu and Arthur, 1992), suggesting that the total cell cycle duration of MCF-7 cell is 20 h. Exponentially growing MCF-7 cells are randomly distributed in the cell cycle with a population of 55%, 25% and 20% of the cells at G1, S and G2/M are respectively (Figure 20A). The length of each phase is the total cell doubling time multiplied by the proportion of the exponentially growing cells in that phase (Gray and Coffino, 1979). Thus, the duration of G1, S and G2/M phase of MCF-7 cells were estimated to be 11 h (20 h X 55%), 5 h (20 h X 25%) and 4 h (20 h X 20%) respectively. These values were utilized in developing the synchronization protocols for our studies.

We succeeded in obtaining a high population (90%) of cells synchronized at the G1/S boundary by exposing the cells twice to hydroxyurea. Hydroxyurea is a ribonucleotide reductase inhibitor which arrests cells in S phase by decreasing cellular dNTP levels (Adams and Lindsay, 1967; Skoog and Nordenskjöld, 1971) but has no effect on the progression of G1 and G2/M cells. It has been widely used to synchronize cells at the G1/S boundary. The first 16 h incubation period which is longer than the sum of the G2/M and the G1 phases (15 h) was selected so that cells in early G2 phase would have enough time to progress through the cell cycle and accumulate at the G1/S boundary. The subsequent incubation period with 10% FBS-supplemented medium without hydroxyurea was to allow all cells at the G1/S boundary and in S phase to progress through S phase but avoid reentry of cells into a new S phase. This led to the choice of a 13 h incubation period which is less than the total length of the estimated G2/M and the G1 phase (15 h). The advantage of the double hydroxyurea treatment over the single hydroxyurea treatment is that the double hydroxyurea treatment blocks approximately 90% of the cells at a single point of the cell cycle, the G1/S boundary

(Figure 20D), while the single hydroxyurea treatment arrests only 70% of the cells at G1/S boundary with 25% in S phase (Figure 20B). Release of synchronized cells from the second hydroxyurea block resulted in more than 80% of the cells progressing in synchrony along the cell cycle as demonstrated by flow cytometric analysis (Figure 21 and Figure 22). Flow cytometric analysis of synchronized cells released from the G1/S boundary revealed that the total cell cycle duration of MCF-7 cells is approximately 21 hours. While S, G2/M and G1 phases are approximately 5.5, 5 and 10.5 h respectively (Figure 22). These values were similar to those estimated from the doubling time and the cell cycle distribution of the exponentially growing MCF-7 cell.

The second synchronization method utilized a combination of hydroxyurea and nocodazole to synchronize approximately 90% the cells in mitosis (Figure 22). Nocodazole, a well established synchronization agent (Zieve *et al.*, 1980). is a microtubule disrupting agent which prevents the formation of the mitotic spindle and therefore arrests cells in mitosis (Zieve *et al.*, 1980). Since the total cell cycle duration of MCF-7 cells was approximately 21 h, exponentially growing cells should be incubated with nocodazole for at least 21 h so that the majority of the cells would progress to mitosis. However, prolonged treatment of cells with nocodazole is cytotoxic (Zieve *et al.*, 1980). To reduce the length of the incubation time with nocodazole, the cells were pre-synchronized at the G1/S boundary and in S phase with hydroxyurea (Figure 23B). The medium was then replaced with 10% FBS-supplemented medium for 6 h to allow the cells to progress towards mitosis but without entry of the cells in G1 phase (Figure 23C). The addition of nocodazole for 6 h successfully synchronized approximately 90% of the cells at mitosis (Figure 23D).

4.2 Phospholipid fluctuation during the cell cycle

Cells must double their membrane mass prior to cell division in mitosis, hence the phospholipid mass is also expected to double during the cell cycle. However, whether this occurred continuously in a progressive manner throughout the cell cycle or whether this occurred during discrete phases of the cell cycle remained largely unknown (Jackowski, 1994 and 1996). In rat thymocytes, a greater increase in phospholipids mass was observed between 12 to 24 h after mitogenic stimulation than during the first 12 h suggesting that phospholipid accumulation occurs sometime after cells exit the G1 phase (Gross *et al.*, 1988). Other studies reported a decrease in nuclear phospholipids (Maraldi *et al.*, 1993) and nuclear inositol lipids (York and Majerus, 1994) associated with DNA replication. At the time of initiation of our studies, the most comprehensive study to investigate how phospholipids accumulate during the cell cycle was that by Jackowski (Jackowski, 1994). In this study, exponentially growing BAC1.2F5 cells, a macrophage cell line that required colony-stimulating factor-1 (CSF-1) for growth, were synchronized at G0 by CSF-1 starvation. The cells were subsequently stimulated to reenter the cell cycle by the addition of CSF-1 and phospholipid accumulation was assessed by determining the levels of incorporation of ^{32}P in the organic phase of cell extracts. These studies led to the conclusion that phospholipid accumulation in BAC1.2F5 cells occurs in S phase (Jackowski, 1994).

In our study, we synchronized cells at the G1/S boundary or in mitosis with chemical agents which when removed allows us to obtain large numbers of cells synchronized at different stages of the cell cycle. Using a very sensitive method (Zhou and Arthur, 1992), we measured the cellular phospholipid mass of cells at different stages of the cell cycle. As

we reported in the results section (section 3.2), following the release of cells from the G1/S boundary, there was a decrease in the levels of PC, PE and PI by 25.8%, 22.5% and 19.3% respectively during the S phase which was followed by an increase of 78.6%, 49.2% and 53.9% in the G2/M phase respectively (Figure 25). The level of PC, PE and PI decreased by 20.0%, 24.1% and 23.0% respectively as cells entered G1 phase (Figure 25). This led us to conclude that net accumulation of phospholipids in cellular membranes occurs in the G2/M phase. By repeating the studies with cells synchronized at mitosis with nocodazole, we were able to confirm that the observed changes in cellular phospholipid levels were a true cell cycle dependent event rather than a consequence of incubating the cells with hydroxyurea. We observed a 34.8%, 34.0% and 32.9% decrease in the levels of PC, PE, and PI respectively within the first 3 h following removal of nocodazole from the synchronized mitosis cells (Figure 26). A slight decrease in the levels of phospholipids was observed in S phase followed by a 43.8%, 50.3% and 36.2% increase during the G2/M phase respectively (Figure 26).

The results from our studies with MCF-7 cells synchronized at G1/S boundary or mitosis both led us to conclude that phospholipid accumulation occurred in the G2/M phase. Clearly this is significantly different from the conclusion of Jackowski that net phospholipid accumulation in BAC1.2F5 cells occurs in S phase (Jackowski, 1994). While the reasons for the different conclusions are not readily apparent, there are a number of possibilities. The two studies used different cell types. While we used a human breast epithelial cell line for our studies, Jackowski used a colony-stimulating factor-1 (CSF-1) dependent macrophage cell line. It is possible that the mechanism that regulate the accumulation phospholipids in

the cell cycle are cell-type specific. To resolve this, studies would need to be repeated with other cell types.

Another major difference between our studies and those of Jackowski (Jackowski, 1994) was the method of assessment of the phospholipid levels in the cell. While the phospholipid mass in MCF-7 cells was determined by assaying the mass of the individual phospholipid classes using a sensitive analytical procedure, the phospholipid level in BAC1.2F5 cells was estimated by measuring the accumulation of ^{32}P in the organic phase of the cell extracts. The latter procedure does not provide the phospholipid mass per cell but only the amount of radioactivity present in the organic phase of the cell extracts, which may not necessarily translate into mass equivalents. For example, phosphatidylinositol can undergo phosphorylation reactions to produce phosphoinositides which would increase the levels of ^{32}P incorporated into the lipid fraction without increasing the mass of the lipids. Indeed, since there is active metabolism of nuclear phospholipid (Maraldi *et al.*, 1993) and nuclear inositol lipids (York and Majerus, 1994) in S phase, the enhanced incorporation of ^{32}P in organic phase of the cell extracts in S phase observed by Jackowski may not be a reflection of increased phospholipid mass but could be partly due to the enhanced turnover of inositol lipids.

It is worth pointing out that in spite of the different conclusions on the accumulation of phospholipids in MCF-7 cells and BAC1.2F5 cells, both studies revealed that accumulation of phospholipid is not a continuous process throughout the cell cycle but rather it occurs at discrete phases of the cell cycle.

In addition to the increased accumulation of phospholipids in G2/M phase observed

in MCF-7 cells, we also observed a decrease in phospholipid levels at two points in the cell cycle. There was a decrease in early S phase and early G1 phase (Figure 25 and 26). The decrease in cellular phospholipid levels as cells exit mitosis was anticipated since cell division results in a division of the cellular membranes in the parental cell into the two daughter cells. One would in fact have expected the phospholipid levels to decrease by 50%, instead, our study revealed a 35% decrease in cells released from the mitosis block and a 24% decrease in cells released from the G1/S blockage. The lower than expected drop in phospholipid levels in the post mitosis cells may be related to the fact that the earliest samples were obtained 3 h after mitosis when the cells would have progressed significantly along the cell cycle in G1 phase. Under these circumstances, rapid synthesis and accumulation of phospholipid in early G1 phase could mask the decline expected as a consequence of cell division. The reason for the smaller decreases in phospholipid levels in post mitotic cells derived from the G1/S synchronized cell (24%, Figure 25) relative to mitosis synchronized cells (35%, Figure 26) is probably due to the time dependent progressive loss of synchronized cell movement along the cell cycle. Flow cytometric analysis revealed that although most of the cells released from the G1/S boundary were in G1 phase after 14 h, about 20 - 30% of the cell were still in G2/M phase (Figure 22). In contrast, only about 5% of the cells remained in G2/M phase 3 h after removal of the mitosis block (Figure 24). Hence the contamination of the early G1 cells by the significant minority of the G2/M phase cells with their large lipid content would increase the lipid content of the cells resulting in higher levels than those observed in the cells obtained after release from mitosis.

Our studies also revealed that the relative levels of PC, PE and PI did not vary significantly during the cell cycle whether the cells were synchronized in mitosis or in G1/S boundary (Table 4). This observation suggests that there may be coordinated regulation mechanisms responsible for maintaining the levels of these phospholipids, and therefore the phospholipid composition, during the cell cycle.

4.3 Phospholipid metabolism during the S and G2/M phases

As discussed above, the levels of the major phospholipids in MCF-7 cells, PC and PE, undergo changes at discrete phases of the cell cycle. PC, and PE levels increased during the G2/M phase and declined in early S phase and early G1 phase. While cell division is primarily responsible for the decreased levels in the cells as they exited M into G1, the changes observed in S and G2/M phases can only be the consequence of changes in the rate of synthesis and/or catabolism of phospholipids. We therefore investigated the changes in the biosynthesis and catabolism of phospholipids during S and G2/M phases to determine if these could be correlated with the fluctuation of phospholipid levels in the cell cycle. In these studies, we focused on the metabolism of PC, the major phospholipid in MCF-7 cells, primarily because reagents were available for the studies. The results are discussed below.

4.3.1 Phospholipid biosynthesis during S and G2/M phase

The synthetic rate of PC at different phases of the cell cycle was assessed by measuring the incorporation of [^{3}H]choline into PC. While the rate of PC synthesis in early S phase was similar to that of cells at the G1/S boundary, there was an increase in the

incorporation of [^{3}H]choline into PC from late S phase and throughout G2/M phase (Figure 28). The results suggested that HU did not affect PC biosynthesis while the rate of PC synthesis was increased in late S phase and the high rates of synthesis were maintained throughout G2/M. Although the enhanced [^{3}H]choline incorporation into PC in G2/M phase could result from a decrease in cellular choline levels, the fact that there is excessive choline in the medium (4mg/L) coupled with the lack of evidence in the literature that cellular choline level fluctuates in the cell cycle suggests that this is unlikely.

We also observed enhanced incorporation of [^{3}H]choline into P-Cho and CDP-Cho in late S and G2/M (Figure 29) which could contribute to the increased incorporation of [^{3}H]choline into PC. The increase [^{3}H]choline incorporation into P-Cho and CDP-Cho from S and G2/M phase suggest that there is any increase in the activity of choline kinase and PCCT. Because PCCT is the rate limiting enzyme for PC biosynthesis, we measured its activity in membrane fractions isolated from cells at different stages of the cell cycle. The results revealed that both the specific activity and total membrane activity of PCCT were elevated from mid S and throughout the G2/M phase (Figure 31 and 32). Similar results were obtained when the activity of CPT, the enzyme that catalyses the final step in PC synthesis was measured. Together, the results indicate that the enhanced synthesis of PC was the result of activation of PCCT, CPT and perhaps choline kinase; while choline kinase activity was not assayed directly, the increase can be inferred from the enhanced P-Cho synthesis.

Of the three biosynthetic enzymes, we conducted studies to determine the mechanism responsible for the activation of PCCT, the rate limiting enzyme of PC biosynthesis (Kent,

1995 and 1997; Vance, 1991). As activation of PCCT *in vivo* correlates with its translocation from an inactive soluble form to an active, membrane-associated species (Vance, 1989; Kent, 1995), we measured microsomal PCCT levels at different stages of the cell cycle. We were able to demonstrate that the level of microsomal PCCT was elevated during S phase, remained relatively constant in the G2/M phase and decreased to the original level as the cells exited mitosis into G1 phase (Figure 36). The increased levels of PCCT associated with G2/M membranes would be expected to lead to activation of the enzyme. Thus the increased membrane levels would explain the enhanced PCCT activity observed in late S and G2/M phases (Figure 31). This results are consistent with previous finding that PCCT was depleted from the nuclear compartment during S phase and subsequently re-entering the nucleus in late S phase (Hunt and Burdge 1998, Hunt *et. al.*, 1998).

Our results also indicate that the activity of the enzyme is not simply a function of the membrane-associated levels. This stems from the observation that the large decrease in the membrane PCCT level observed in early G1, 13 and 16 h after the cells were released from the G1/S boundary (Figure 36), did not translate into proportionate decreases in the specific activity. These results therefore implicate other processes in the regulation of PCCT activity. Phosphorylation could be such an event but the role of phosphorylation in regulating the catalytic activity of PCCT in the cell cycle is unclear even thought the enzyme has numerous phosphorylation sites that are potential targets of cyclin-dependent kinase (Cdks) (Wieprecht *et al.*, 1996). The oscillation of PCCT activity in the cell cycle suggest that Cdk might regulate PCCT activity via phosphorylation. Indeed, Jackowski showed that the extent of PCCT phosphorylation fluctuates during the cell cycle (Jackowski, 1994). It is also worth

knowing that PCCTα is an intranuclear protein (Wang *et al.*, 1993b) and the active form of PCCT localizes to the nuclear membrane (Watkins and Kent, 1991; Wang *et al.*, 1993a; Wang *et al.*, 1993b). This places PCCTα in the correct subcellular compartment for regulation by cyclin-dependent kinases which undergo cell cycle dependent activation and nuclear translocation (Pines, 1995). Although regulation of PCCT activity by cyclin-dependent kinase phosphorylation has not been directly demonstrated, it has been reported that PCCT is phosphorylated *in vitro* by cyclinB/Cdc2 kinase (Cornell *et al.*, 1995; Wieprecht *et al.*, 1996). Our attempts to assess the phosphorylation state of the enzyme with phospho-Ser antibodies were unsuccessful due to the lack of specificity displayed by the antibodies (data not shown). The idea that Cdks might control the pace of PC synthesis and hence membrane synthesis in the cell cycle through the direct phosphorylation of PCCT still remains to be demonstrated.

As pointed out above, CPT activity was also activated in tandem with PCCT activity, however, we are unable to investigate the mechanism responsible for its activation due to the lack of agents, such as antibodies. Indeed very little is known about the regulation of CPT activity *in vivo*.

Another observation worth discussing is that the activities of both PCCT and CPT in fractions isolated from cells at the G1/S boundary were significantly lower than the activities in other points of the G1 phase (Figure 31 and 32). The reduced activity was not due to inhibition of the enzyme activity by hydroxyurea, the cell synchronization agent (Figure 33). With respect to PCCT, we can infer that the reduced activity is not due to lower amounts of membrane associated enzyme (Figure 36). From these observations, it is

reasonable to suggest that as cells approach the G1/S boundary there is a significant inhibition of the activities of PCCT and CPT. The basis for this inhibition is not known. It should be pointed out that not all the enzymes we assayed exhibited this phenomenon, for example, PECT activity was unaffected. It is tempting to speculate that the decreased activity of PCCT and CPT at G1/S boundary and S phase contribute to the reduced incorporation of [^{3}H]choline into PC and the decrease in PC levels observed in early S phase.

Overall, the results of the studies on changes in PC synthesis during the cell cycle indicate that the changes correlate with fluctuations of PC levels in the cell. Thus we conclude that the increased levels of PC in the MCF-7 cell during the G2/M phase occur in part from enhanced synthesis while decreased synthesis contribute to the reduced PC levels observed in early S phase.

The rate of PE biosynthesis along the cell cycle was investigated by assaying the incorporation of [^{14}C]ethanolamine into PE. The pattern of incorporation of the label into PE was quite distinct from that observed for PC. Incorporation of [^{14}C]ethanolamine into PE was relatively similar at all times except for an increase in late S phase, suggesting that the rate of PE synthesis in the cell cycle was relatively constant (Figure 30). Fluctuations in the cellular ethanolamine levels during the cell cycle could interfere in the incorporation of label into PE. Thus, if the level of ethanolamine increases in G2/M phase, the specific activity of ethanolamine would decrease leading to a decline in the incorporation of ethanolamine into PE during G2/M phase. However, there is no evidence in the literature that the cellular levels of ethanolamine fluctuate in the cell cycle.

Analysis of the activity of PECT, the rate limiting enzyme in PE synthesis again

showed little change in the activity up to mid-G2/M phase. There was a slight increase in late G2/M phase. In contrast, EPT, the enzyme that catalyzes the condensation of DAG with the phosphoethanolamine moiety of CDP-Etn to form PE, had a pattern similar to that observed for PCCT and CPT. EPT activity increased significantly and progressively throughout S and the G2/M phase. Since the rate of [^{14}C]ethanolamine incorporation into PE was more in tune with the PECT profile rather than EPT profile, our results would indicate that the contribution of EPT activity to the overall regulation of PE synthesis is not major. Even at early S phase, the EPT activity is more than enough to convert all the CDP-ethanolamine to PE. The lack of correlation of PE synthesis with the accumulation of PE would suggest that modulation of *de novo* PE synthesis is unlikely to be responsible for the observed accumulation of PE in G2/M phase. This result suggests that cessation of PE catabolism and/or enhanced PE biosynthesis from decarboxylation of phosphatidylserine may be responsible for the observed PE accumulation in the cell cycle.

4.3.2 The role of CTP in phospholipid biosynthesis during the cell cycle

As CTP is a common precursor required for the synthesis of all phospholipids, we investigated whether fluctuation in CTP levels during the cell cycle could contribute to the changes in phospholipid synthesis observed. Our results revealed that except for a 20% decrease during early S phase, the CTP levels were unchanged throughout the cell cycle (Figure 37). The coincident decrease in phospholipid content and CTP levels in early S phase suggest that the two are related. This idea could be valid as far as PC levels are concerned, since the decreased levels coincided with diminished synthesis. However, it is

unlikely to be valid for PE, because there was no decrease in the rate of PE synthesis which would be expected if the lower CTP levels significantly affected the rate of CDP-Etn synthesis. Differential regulation of PC and PE synthesis by CTP is feasible since PCCT is thought to be nuclear enzyme which binds to the nuclear membrane (Kent, 1995 and 1997), while PECT is cytosolic (Vermeulen *et al.*, 1997). Compartmentalisation of CTP in the cell would therefore differentially regulate the synthesis of phospholipids. Any future studies to investigate the role of CTP in regulating phospholipid synthesis would need to measure the levels of CTP in different cellular compartments. In addition, a clearer appreciation of the impact of CTP levels on phospholipid synthesis would be obtained if the CTP concentrations, rather than the cellular CTP contents, were known. This is because changes to the cell volume occur during the cell cycle. Hence, even though the absolute CTP levels may not change, an increase in cell volume would have a diluting effect on CTP concentration which could in turn affect the rate of phospholipid synthesis. Further studies would need to monitor the cellular volume in the cell cycle in order to be able to estimate the concentration of CTP and clarify the effect, if any, that concentration changes have on lipid synthesis in the cell cycle.

4.3.3 Phospholipid catabolism in the S and G2/M phases

Since cellular phospholipid levels reflect the relative rates of synthesis and catabolism, it was important to investigate the contribution of phospholipid catabolism to the changes in the levels of phospholipid observed during the cell cycle. The results of the studies discussed above on the contribution of synthesis to fluctuation in the phospholipid

levels in MCF-7 cells indicated that regulating the rate of phospholipid catabolism could be very significant in regulating the accumulation of PE in the cell cycle. This is because the synthetic rate and specific activity of PECT, the rate limiting enzyme in PE synthesis were similar during most parts of the cell cycle. On the other hand, we detected increased PC synthesis at regions in the cell cycle where there was enhanced PC accumulation and decreased PC synthesis where PC levels declined.

Attempts to study PC and PE catabolism by pulse-chase studies with [^{3}H]choline and [^{14}C]ethanolamine were unsuccessful because labelling of the large phosphocholine and phosphoethanolamine pools resulted in continuous incorporation of label into the PC and PE during the chase. It was therefore not feasible to study the loss of label from the phospholipid at specific positions in the cell cycle. This led to the use of radiolabelled lysophospholipids to label phospholipids, an approach that has been used as a means to circumvent the labeling of phospho-base pools (Baburina and Jackowski, 1999). Since only radiolabelled lysoPC was available, our studies were confined to investigating the catabolism of PC during the MCF-7 cell cycle.

The use of radiolabelled lysoPC to label cellular PC clearly has advantages over the use of the labelled choline in the catabolic studies. However, as lysoPC is incorporated into PC via acylation, there may be disadvantages with respect to whether one labels specific pools as opposed to all the cellular pools which one would expect to be labelled with choline which is incorporated via the *de novo* synthetic pathway. The 3 h pulse selected to label MCF-7 cells with [methyl-^{3}H]lysoPC was chosen because equilibrium labelling had been achieved (Figure 38). The reason for the rapid elevation of radioactivity associated with PC

without a corresponding decrease of cellular [methyl-^{3}H]lysoPC is because of the high lysoPLase activity in cell. Since lysoPC has detergent properties and is cytolytic at high concentrations (Weltzien 1979), cellular lysoPC has to be kept at very low level. Only a minor amount of the lysoPC incubated with the cells was converted to PC while the majority was hydrolyzed in the medium by lysophospholipase (Figure 43). The much greater rate of accumulation of label in medium GPC compared to accumulation in cellular GPC and PC clearly showed this was the case. This interpretation was also supported by the results of studies with [1-palmitoyl-^{14}C]lysoPC (Table 10). We demonstrated that lysoPC was chemically stable and also that the 10% FBS medium did not contain lysoPC-hydrolyzing lipase activity. It is presumed that the lysophospholipases are on the surface of the cell and can thus access the substrate in the medium. Since [methyl-^{3}H]GPC was not taken up by the cells, there was no problem with recycling of the label from the metabolite back into the cells. On the other hand, when [1-palmitoyl-^{14}C]lysoPC was used, some of the medium FA produced by lysophospholipase activity was taken up into the cells and incorporated into PC and PE. This would explain the lower recovery of labelled FA recovered (45%) in the medium compared to labelled choline in GPC (70%) after 3 h incubation of the cell with the lysoPCs (Table 9 and 10). It would also account for the fact that more radioactivity was found in PC when cells were incubated with [1-palmitoyl-^{14}C]lysoPC than with [methyl-^{3}H]lysoPC (Table 9 and 10).

Even in experiments where the cells were incubated with [methyl-^{3}H]lysoPC and washed prior to chasing with 10% FBS-containing medium, the major catabolite was the medium GPC (Table 11). This metabolite could arise from the conversion of lysoPC to PC

in the cells followed by degradation of the PC to GPC and its secretion from the cell into the medium (Kwon *et al.*, 1995 and 1996; Baburina and Jackowski, 1999). In the experiments with [1-palmitoyl-^{14}C]lysoPC designed to test this notion, we were unable to confirm the accumulation of the expected palmitoyl-containing metabolites in the cells but instead we noticed that the hydrolysis of PC resulted in the accumulation of FA in the medium (Table 12). These observations would be consistent with the translocation of PC from the cells to the outer lipid bilayer, where it was hydrolyzed sequentially by phospholipases and lysophospholipases. There is evidence that after synthesis of PC at the inner leaflet of the lipid bilayer, PC is quickly translocated to the outer leaflet of the lipid bilayer (Andrick *et al.*, 1991; Bitbol and Devaux, 1988). Clearly, this is required to maintain the asymmetry of the membrane lipid bilayer (Op den Kamp 1979, Alberts *et al.*, 1994a). In light of the above, the possibility that the protocols used in our catabolic studies may only measure the degradation of plasma membrane PC cannot be discounted. However, the duration of the lysoPC pulse used in our studies which resulted in equilibrium labelling and evidence from immunofluorescent studies that show that plasma membrane lipids are rapidly internalized and distributed to intracellular membranes (Sleight and Pagano, 1984 and 1985), lead us to suggest that the results obtained with our protocols reflect the aggregate degradation of the entire cellular PC pool.

We investigated the catabolism of PC during S and G2/M phases in MCF-7 cells by synchronizing cells pre-incubated with [methyl-^{3}H]lysoPC at the G1/S boundary and chasing with 10% FBS-supplemented medium. Our studies revealed that the radioactivity associated with PC decreased rapidly in S phase (23% decrease in the first 4 h of S phase) with very

little loss of label in the G2/M phase (Figure 42). The cessation of PC catabolism in G2/M phase was confirmed by labelling cells in S phase and chasing in G2/M (Figure 42). In contrast, the loss of label from PC in exponentially growing MCF-7 cells was linear throughout the chase period (Figure 41 and Figure 42), indicating that the profile obtained with the synchronized cells reflected the different rates of PC catabolism occurring in the cells at different stages of the cell cycle. The coincidence in the increase in PC degradation and diminished PC levels in early S phase suggest that the later is a consequence of the former. We can therefore conclude that the enhanced PC catabolism contributes to the reduced PC levels observed in S phase, while the cessation of PC catabolism in G2/M phase also contributes to the observed PC accumulation in G2/M phase.

The biological significance of the enhanced phospholipid degradation during S phase is not known. However, the enhanced phospholipid catabolism in S phase could generate signalling molecules that function to initiate and/or stimulate DNA replication (Maraldi *et al.*, 1993; York and Majerus, 1994; Dowhan, 1997). The diacylglycerol released by PLC may activate nuclear protein kinase C which has been found to phosphorylate and activate DNA polymerase and topoisomerase (Divecha *et al.*, 1991; Fields *et al.*, 1990; Divecha *et al.*, 1997). Ins(1,4)P_2 binds DNA polymerase α and results in an increased affinity of the enzyme for DNA template/primer and a 10-fold increase in enzyme activity (Sylvia *et al.*, 1988), suggesting that inositol polyphosphates may play a role in DNA replication. The identification of an inositol 1,4,5-trisphosphate receptor in the inner nuclear membrane that mediated calcium release into the cytoplasm (Humbert *et al.*, 1996) indicates that inositol polyphosphates may play a role in regulating nuclear calcium concentration which in turn

could influence DNA replication and gene transcription (Carafoli et al., 1997). The enhanced phospholipid breakdown may also generate FA and DAG which in turn activate PCCT and thus, result in enhanced PC biosynthesis and PC accumulation in G2/M phase. It is probably unlikely that all the phospholipid catabolites produced in S phase in our studies participate in the signalling functions discussed above.

The separation of DNA biosynthesis and the production of membrane phospholipid at different phases of the cell cycle may serve the purpose of limiting the massive amount of the cellular energy and CTP that would otherwise be required if both DNA replication and membrane phospholipid production occurred in the same phase.

5 CONCLUSION

We have investigated the coordination and regulation of membrane phospholipid accumulation in MCF-7 cell cycle. Using two cell synchronization procedures that we developed, we successfully obtained large populations of synchronized cells at different stages of the cell cycle. Measurement of phospholipid content in the cell cycle revealed that PC, PE and PI content decreases rapidly during S phase followed by an accumulation in G2/M. But the composition of PC, PE and PI remained relatively constant along the cell cycle, suggesting that major phospholipid production is coordinately regulated in the cell cycle.

The contribution of both synthesis and catabolism to the observed phospholipid fluctuation during S and G2/M phases were investigated. Our studies demonstrated that the decrease in PC content during S phase resulted from an enhanced PC degradation and a relative low synthetic rate, while PC accumulation in G2/M was attributed to both enhanced PC biosynthesis and the cessation of PC degradation. The rate of PE synthesis and the activity of PECT, the rate limiting enzymes in PE biosynthesis, were not significantly changed during S and G2/M phase, suggesting that PE catabolism may be responsible for the observed PE fluctuation during S and G2/M phases. The changes of cellular CTP levels did not appear to regulate phospholipid accumulation during the cell cycle.

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