Homeostasis of phosphatidylcholine (PC) is regulated by the opposing actions between CTP:phosphocholine cytidylyltransferase (CT) and the group VIA Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)). We investigated this process during the cell cycle. PC mass doubles during late G\(_1\) and early S phase when its rate of catabolism is lowest. We show that iPLA\(_2\) activity is cell cycle-dependent with peak activity during G\(_2\)/M and late S phase. iPLA\(_2\) activity declines during G\(_1\) and is lowest at the G\(_1\)/S transition and early S phase. The accumulation of PC correlates with decreased iPLA\(_2\) activity, suggesting that regulation of this enzyme contributes to phospholipid accumulation. The levels of 80 kDa iPLA\(_2\) protein do not change and thus cannot account for changes in enzyme activity. Reverse transcriptase and real-time PCR experiments show that splice variant iPLA\(_2\) mRNAs are preferentially expressed during G\(_2\)/M. Immunoblot analyses with an antibody directed against the N terminus of iPLA\(_2\) revealed a ~50 kDa protein that is of appropriate size to be the truncated protein encoded by the ankyrin-iPLA\(_2\)-1 splice variant mRNA. The levels of truncated iPLA\(_2\) protein were high in cells in late G\(_1\) and S phase cells that had low iPLA\(_2\) activity and low in G\(_2\)/M cells that had high iPLA\(_2\) activity. The truncated protein co-immunoprecipitated with full-length iPLA\(_2\), indicating a physical interaction between the two proteins. Together, these data suggest that truncated iPLA\(_2\) proteins associate with active iPLA\(_2\) and down-regulate its activity during G\(_1\). This down-regulation may contribute to phospholipid accumulation during the cell cycle.

Eukaryotic cell membranes are composed of a complex collection of glycerophospholipids, sphingosylphospholipids, proteins, and cholesterol. Phosphatidylcholine (PC)\(^1\) is the most abundant membrane glycerophospholipid in mammalian cells and thus serves as an important structural element of cell membranes. In addition to its importance with respect to membrane structure, it also serves as a precursor for the synthesis of sphingomyelin (1), phosphatidylethanolamine (2), and a variety of bioactive lipid signaling molecules that regulate cell behavior (3). Tightly coordinated regulatory mechanisms are necessary to maintain PC homeostasis. Several reports have suggested that in some cell types PC homeostasis is maintained through the opposing actions of CTP:phosphocholine cytidylyltransferase-\(\alpha\) (CT\(\alpha\)) and the group VIA Ca\(^{2+}\)-independent phospholipase A\(_2\) (iPLA\(_2\)). CT\(\alpha\), the rate-limiting enzyme in the major pathway for PC synthesis in mammalian cells (4), catalyzes the formation of CDP-choline from CTP and phosphocholine. iPLA\(_2\) is an sn-2 directed acylhydrolase that catalyzes glycerophospholipid into free fatty acid and 2-lysophospholipid. Overexpression of CT\(\alpha\) in COS, CHO-K1, and HeLa cells increased PC synthesis, yet PC mass remained relatively constant because of increased catabolism (5–7). We showed that iPLA\(_2\) activity and protein were up-regulated in CHO cells that overexpressed CT\(\alpha\) (5), suggesting that this enzyme was responsible for maintaining membrane phospholipid mass by modulating the rate of PC catabolism. In addition to its role in maintaining glycerophospholipid homeostasis, iPLA\(_2\) is also involved in other cellular processes, including acyl chain remodeling, apoptosis, and eicosanoid production (8–10).

Active iPLA\(_2\) is predicted to be a homotetramer of ~80 kDa monomers (11). A recent report suggests that the catalytic activity of the enzyme is modulated by catalytically inactive truncated iPLA\(_2\) proteins that are encoded by alternatively spliced mRNAs (12). These splice variants are referred to as ankyrin-iPLA\(_2\)-s because the alternative splicing is predicted to generate truncated proteins that retain the N-terminal ankyrin repeats but lack the C-terminal lipase site. These catalytically inactive proteins are thought to disrupt the oligomerization of full-length monomers and thereby down-regulate the activity of the enzyme.

At some point during the cell cycle, the net rate of lipid synthesis must overcome the rate of catabolism to allow for the doubling of lipid mass for daughter cells. This accumulation of PC is essential for cell cycle progression. Decreased PC synthesis and mass occurs when C3H/10T1/2 fibroblasts, WI-38 fibroblasts, and L6 myoblasts are deprived of choline and results in growth arrest during G\(_1\) (13, 14). Reconstitution with choline or lyso-PC rescues these cells and allows cell cycle progression. More recently, it has been shown that inhibition of phosphatidate phosphohydrolase-1 blocks PC synthesis and induces apoptosis in a variety of cell lines (15). Interestingly, although PC accumulation occurs in G\(_1\)/S, the rate of PC synthesis and activity of CT\(\alpha\) are highest in early G\(_1\) (16, 17). These observations indicate that PC accumulation during S phase is not solely dependent on increased synthesis. Instead, several reports suggest that a decrease in phospholipid turnover contributes to the net accumulation of PC during late G\(_1\) and S phase (13, 16, 18, 19). These observations suggest that one or more of the phospholipase activities must be regulated during the cell cycle.

Given its other roles in basal phospholipid metabolism, we were intrigued by the possibility that iPLA\(_2\) activity might be down-regulated to allow phospholipids to accumulate during S phase. To test this hypothesis, we compared iPLA\(_2\) activity and...
expression in cells at each phase of the cell cycle. We demonstrate that iPLA₂ activity is regulated during the cell cycle and that the regulation of iPLA₂ activity may account for glycerophospholipid accumulation during late G₁ and early S phase. Our studies also indicate that the mechanism of this regulation is dependent on alternative splicing of the iPLA₂ gene.

EXPERIMENTAL PROCEDURES

Materials—CHO-K₁ and Jurkat T cells were purchased from ATCC (Manassas, VA). Hydroxyurea and choline chloride were obtained from Sigma-Aldrich. Thymidine, bovine pancreas ribonuclease A, nocardazole, and propidium iodide were all obtained from Calbiochem (San Diego, CA). 1-Palmitoyl-2-[¹⁴C]palmitoyl-2-arachidonyl phosphatidylcholine, 1-palmitoyl-2-[¹⁴C]arachidonyl-3-phosphocholine, 1-palmitoyl-2-[¹⁴C]arachidonyl-3-phosphocholine, [¹³C]oleic acid, and [³H]choline chloride were purchased from New England Nuclear (Boston, MA). Unlabeled lipids were obtained from Avanti Polar Lipids (Alabaster, AL). A rabbit polyclonal anti-iPLA₂ antibody was obtained from Cayman Chemical (Ann Arbor, MI). Another anti-iPLA₂ antibody (P-19) that recognizes an undisclosed epitope within the first 100 amino acids and the corresponding blocking peptide were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-iPLA₂ used for immunoprecipitation (T-14) was obtained from Santa Cruz Biotechnologies and used for immunoblotting experiments as well. Ankyrin-iPLA₂-1 cDNA was a kind gift of Dr. Brian Kennedy (Merrck Frosst Canada & Co.).

Cell Culture and Synchronization—CHO-K₁ and Jurkat T cells were cultured in Ham’s F-12K and RPMI media, respectively, supplemented with 10% heat inactivated fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine. Cells were maintained at 37 °C and 7.5% in a humidified atmosphere. Cell viability was determined using Trypan blue and exceeded 90% in all experiments. For CHO-K₁ synchronization, subconfluent cells were treated with 5 mM thymidine for 24 h. Cells were then released from this block and cultured with fresh medium for 8 h under normal culture conditions. Nocodazole was then added to a final concentration of 0.06 µg/ml for 6 h. A mitotic shake-off was then performed to isolate cells synchronized in G₂/M. For Jurkat T cells, immunoaffinity purification was performed by incubating the cells with a monoclonal antibody against ankyrin-iPLA₂-1 and co-workers (21).

Celluar Triton X-100, with 0.02 µM of t-1-palmitoyl-2-arachidonyl[arachidonyl-1-³H]phosphatidylcholine, 1 micromolar dioleoylglycerol. Assays were processed and the released [³H]oleic acid or [¹⁴C]arachidonic acid was quantified according to the method of Dole (23). The assays were performed under conditions where substrate hydrolysis was linear with respect to protein and time.

Phospholipase D Activity Assay—Phospholipase D activity was assayed by measuring phosphatidylethanol formation (transphosphatidylation). CHO-K₁ cells were labeled for 24 h with 1 µCi/ml [³H]oleic acid. During the last hour of incubation, 1% ethanol was added to the culture medium. After harvesting the cells, the phospholipids were extracted according to the Bligh and Dyer method (24) and radiolabeled phospholipids were quantified following fractionation by thin layer chromatography on Silica Gel G developed with ethyl acetate/isooctane/ acetic acid/water (110:50:20:100 v/v, upper phase) (analysis of phosphatidylethanol or chloroform/methanol/ammonium hydroxide (65:25:5) (stabilization of PC). Lipids were visualized using iodine vapors and subsequently scraped for analysis based on co-migration with known standards. The radiolabel associated with phosphatidylethanol and PC was quantified and % transphosphatidylation was calculated (ratio of dpm in phosphatidylethanol/dpm in PC).

Quantification of PC Mass—Equal numbers of CHO-K₁ cells were homogenized in assay homogenization buffer. Lipids were extracted using the method of Bligh and Dyer (24). PC was separated by TLC on silica plates, the PC spot was identified by co-migration with known standards, and the associated radiolabel was quantified by scintillation counting. The raw data were corrected for extraction and counting efficiencies and were normalized for the total amount of [³H]choline incorporated during the 15 min labeling period. Thus the data are presented as dpm PC/dpm choline.

CT Activity Assays—CT activity was measured in whole cell lysates prepared from synchronized CHO-K₁ cells. Cell homogenates were prepared, and CT activity was quantified by measuring the conversion of ['³H]choline chloride to ['³H]choline. The cells were treated with 100 µg/ml of RNase A (62 µg/ml for 30 min at 37 °C. Cell samples were then stained with 100 µg/ml of propidium iodide. The samples were then analyzed on a BD Biosciences flow cytometer to obtain DNA content profiles. The histograms were analyzed using the ModFitLT cell cycle analysis software from Verity INC.

Immunoblot Analysis—Equal amounts of proteins were separated by SDS-PAGE (5%), electroblotted onto PVDF membranes, and probed for iPLA₂ antibodies by immunoblotting. Immunoblot analyses were performed as described previously (5). The substrate and product were separated by TLC on silica plates with methanol, 0.5% sodium chloride, ammonium hydroxide (50:50:1) as the mobile phase. The phosphocholine and CDP-choline bands were identified based on co-migration with known standards, and the radiolabel associated with each band was quantified by scintillation counting. The specific radioactivity and the conversion efficiencies were normalized for the total amount of [³H]choline incorporated during the 15 min labeling period. Thus the data are presented as dpm PC/dpm choline.

Quantification of PC Turnover—CHO-K₁ cells were pulsed with 2 µCi/ml [¹³C]choline chloride for 15 min during various phases of the cell cycle. After labeling, the cells were washed three times and lipids were extracted with the method of Bligh and Dyer (24). PC was separated by TLC on silica plates, the PC spot was identified by co-migration with a known standard, and the associated radiolabel was quantified by scintillation counting. The raw data were corrected for extraction and counting efficiencies and were normalized for the total amount of [³H]choline incorporated during the 15 min labeling period. Thus the data are presented as dpm PC/dpm choline.

RNA Isolation, Reverse Transcriptase PCR, and Real-time PCR—Jurkat T cells at different stages of the cell cycle were collected and total
mRNA was isolated using TRIzol reagent (Invitrogen). 800 ng of total RNA was reverse-transcribed using Thermoscript reverse transcriptase (Invitrogen) and subjected to 30 cycles of PCR amplification (95 °C for 1 min, 55 °C for 45 s, and 72 °C for 1 min). The iPLA2-specific primers used were 5′-H11032-cagggctctgcagcgccacatcat-3′ (forward) and 5′-H11032-ggccctctcgatggcgagtaggag-3′ (reverse) and they generated 237, 290, and 342 base pair products as expected based on Larsson et al. (12). Quantitative real-time PCR was employed to determine the levels of iPLA2 and ankyrin-iPLA2 transcripts. The probe and primers used to detect full-length iPLA2 mRNA were 5′-H11032-acgagaagcggacccacgaccac-3′ (Taqman probe), 5′-H11032-cgttcatcctgggctccat-3′ (forward), 5′-H11032-tgaggagctggatgatgag-3′ (reverse). These reagents are designed to amplify a product that contains the border between exon 10 and exon 11. The probe and primers used for detection of the ankyrin-iPLA2 mRNA transcripts were 5′-H11032-catgcagaacctgagcccctgagag-3′ (Taqman probe), 5′-H11032-gctccatgagggagagaag-3′ (forward), 5′-H11032-tccgtgtgaccttgagcaa-3′ (reverse). These reagents are designed to amplify a product that contains the border between exon 10 and exon 10a. The probes and primers were designed using the Primer Express™ 1.5 version. The Taqman probe was labeled in the 5′-end with FAM (fluoresceine) and in the 3′-end with TAMRA. The experiments were performed with the ABI Prism® 7700 Sequence Detection System (Applied Biosystems) using the TaqMan® One Step PCR Master Mix Reagents Kit. All samples were tested in triplicate under the conditions recommended by the fabricant. The CT threshold was determined to provide the optimal standard curve values. The signal for iPLA2 mRNA expression was normalized to 18S RNA expression (Pre-developed TaqMan® Assay Reagents).

Cloning and Overexpression of Hamster iPLA2—Total RNA was extracted from CHO-K1 cells using TRIzol reagent. cDNAs were synthesized and amplified using the Thermoscript RT-PCR system (Invitrogen) and PfuUltra polymerase (Stratagene). The full-length hamster iPLA2 was amplified utilizing PCR primers that incorporated 5′-NheI and 3′-ClaI restriction sites (5′-actgctgctagcatgcagttcttcggacgcc-3′ (forward), 5′-gcagggagcagccagccccctctcctg-3′ (reverse)). These reagents are designed to amplify a product that contains the border between exon 10 and exon 11. The probe and primers used for detection of the ankyrin-iPLA2 mRNA transcripts were 5′-catgcagaacctgagcccctgagag-3′ (Taqman probe), 5′-gcagggagcagccagccccctctcctg-3′ (forward), 5′-tccttgggtctgagcagcagcagcagcag-3′ (reverse). These reagents are designed to amplify a product that contains the border between exon 10 and exon 11. The probes and primers were designed using the Primer Express™ 1.5 version. The Taqman probe was labeled in the 5′-end with FAM (fluoresceine) and in the 3′-end with TAMRA. The experiments were performed with the ABI Prism® 7700 Sequence Detection System (Applied Biosystems) using the TaqMan® One Step PCR Master Mix Reagents Kit. All samples were tested in triplicate under the conditions recommended by the fabricant. The CT threshold was determined to provide the optimal standard curve values. The signal for iPLA2 mRNA expression was normalized to 18S RNA expression (Pre-developed TaqMan® Assay Reagents).

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Fluorescence Intensity (FL3)

**FIG. 1.** Propidium iodide staining and FACS analysis of synchronized CHO cells. Cells were synchronized using thymidine and nocodazole as described under “Experimental Procedures.” A, asynchronous control cells (48 ± 6% G1, 18 ± 2% S, 34 ± 7% G2/M). B, CHO cells prior to release from nocodazole block (3 ± 3% G1, 5 ± 5% S, 92 ± 4% G2/M). C, CHO cells 2 h after release from nocodazole block (76 ± 12% G1, 21 ± 15% S, 3 ± 3% G2/M). D, CHO cells 6 h after release from nocodazole block (79 ± 7% G1, 16 ± 3% S, 5 ± 4% G2/M). E, CHO cells 8 h after release from nocodazole block (17 ± 4% G1, 72 ± 8% S, 11 ± 6% G2/M). F, CHO cells 10 h after release from nocodazole block (13 ± 7% G1, 78 ± 7% S, 9 ± 1% G2/M). Cell cycle analysis was performed with ModFit LT cell cycle analysis software from Verity Inc. A representative experiment is shown (n = 3).
instructions. iPLA₂ expression was induced by culturing the transfected cells overnight in culture medium containing 500 ng/ml doxycycline.

Statistical Analyses—For most experiments, the means ± S.E. of a minimum of three determinations are shown. Data were analyzed with Student’s t test with p < 0.05 used as the cut-off for statistical significance.

RESULTS

To understand the process of net phospholipid accumulation during the cell cycle and determine if iPLA₂ contributes to this process, CHO-K1 fibroblasts were synchronized in G₂/M using thymidine and nocodazole (20). A mitotic shake off was performed, and the synchronized cells were then re-plated and allowed to progress through the cell cycle under normal culture conditions. Propidium iodide staining and flow cytometric analyses were performed to monitor the progression of the cells through the cell cycle (Fig. 1). An asynchronous (asyn) population of cells was used as a comparative control (Fig. 1A). Immediately after release from the mitotic block, over 90% of the cells were in G₂/M (Fig. 1B). Cell division was complete within ~45 min after the nocodazole was washed from the cells. The cells were determined to be in G₁ from 1 to 7 h after release from nocodazole (Fig. 1, C and D). The histograms in Fig. 1, E and F indicate that S phase begins at 8 h and lasts through 10 h after the release from nocodazole. Beyond 10 h of incubation, the cells were no longer highly synchronized (data not shown). Thus, this method allowed study of CHO-K1 cells in G₂/M, throughout G₁, and in S phase.

CHO cells synchronized in G₂/M contained 1.72 ± 0.12 µg of lipid phosphate per 2.5 million cells (Fig. 2A). Immediately after cell division PC mass was reduced by ~50%, consistent with partitioning of lipid to the membranes of daughter cells. PC mass increased during G₁ and reached G₂/M levels by early S phase. These results demonstrate that PC mass accumulates before cell division and are consistent with previous reports (16, 26).
As several reports have attributed glycerophospholipid accumulation to decreased turnover (16, 18), we measured the half-life of PC to determine if this was true in the CHO cell system as well. To achieve this, CHO cells were labeled to equilibrium with [3H]choline, arrested at various points in the cell cycle, and then subjected to a chase with excess unlabeled choline. The thymidine and nocodazole synchronization method described for PC mass analysis was used to generate cells in G2/M or mid-G1. However due to the complications associated with maintaining cells at the border of G1 and S phase during the long choline chase period, we used 5 mM hydroxyurea for 12 h to achieve synchronization in G1/S. This method successfully synchronized these cells in G1/S as determined by FACS analysis of DNA content (data not shown). As shown in Fig. 2B, the half-life of PC was ~3-fold longer during G1/S than it was during G2/M and the G1 half-life was between these two values but this difference was not statistically significant. Thus, the rate of PC turnover is highest during G2/M and lowest at the G1/S boundary. The decline in PC turnover at the G1/S boundary is consistent with previous reports and indicates that this allows for net PC accumulation before cell division.

It is well established that PC accumulation during the cell cycle does not correlate with the kinetics of its synthesis (16, 26, 27). We obtained similar results in the CHO cell system as the cells exhibited a burst of PC synthesis but this occurred in early G1 and not during late G1 and S phase when PC mass accumulated (Fig. 3A). Furthermore, the catalytic activity of CT, the rate-limiting enzyme in PC synthesis, was relatively static throughout the cell cycle (Fig. 3B). Taken together, these results suggest that net accumulation of PC during the cell cycle is not strictly dependent on its rate of synthesis but is largely mediated through decreased catabolism.

CHO cells express a variety of enzymes that catabolize PC,
including iPLA₂, the group IVA calcium-dependent cytosolic PLA₂-α (cPLA₂), and phospholipase D (PLD). To determine if one or more of these enzymes were regulated to allow for PC accumulation, we assessed their activities in CHO cells at each stage of the cell cycle. Cells were harvested at various stages of the cell cycle and phospholipase activities were measured as described under "Experimental Procedures." As shown in Table I, the catalytic activity of cPLA₂ does not change during the cell cycle. PLD activity is cell cycle-dependent, but is highest during S phase and therefore does not correlate with the increase in PC half-life observed at this time (Fig. 2B).

Thus, neither cPLA₂ nor PLD activities appeared to account for the decline in turnover that allowed PC to accumulate during the cell cycle.

In contrast, iPLA₂ activity varied by nearly 3-fold during the cell cycle (Fig. 4A). iPLA₂ activity was highest during G2/M and late S phase and steadily declined during G1 until it reached its lowest point at the G1/S border (≈8 h after release from nocodazole). Activity increased during S phase and returned to peak levels as the cells prepared to enter G2. Thus, iPLA₂ activity correlates with the kinetics of PC turnover, suggesting that this enzyme may be down-regulated to allow for phospholipid accumulation through decreased catabolism. To confirm that iPLA₂ activity is lower at G1/S than G2/M, we treated CHO cells with 5 mM hydroxyurea for 12 h to arrest the cells at G1/S and compared iPLA₂ activity in these cells with cells synchronized in G2/M. As expected, iPLA₂ activity was ≈50% lower in G1/S cells than in G2/M cells (Fig. 4B).

We next investigated the regulatory mechanism that allowed for the observed changes in iPLA₂ activity during the cell cycle. The simplest and most obvious explanation is that iPLA₂ protein mass is cell cycle-dependent and lowest in cells at G1/S. However, immunoblot analyses (Fig. 4A, inset and B, inset) indicated that iPLA₂ protein mass was similar at all phases of the cell cycle, suggesting that simple changes in protein mass cannot account for the changes in iPLA₂ activity.

Alternative splicing of the human iPLA₂ gene has been predicted to generate truncated iPLA₂ proteins that can negatively regulate enzyme activity (12). Similar to our observations in the CHO cell model, human Jurkat T cells have cell cycle-dependent iPLA₂ activity, with lowest activity at G1/S. As genomic information is not available for hamster iPLA₂, Jurkat was a convenient model to study the regulation of iPLA₂ during the cell cycle. Using nocodazole, hydroxyurea, and thymidine, we synchronized Jurkat T cells in G2/M, G1/S, and S phase respectively according to a previously established method (21). As shown in Fig. 5A, this approach produced highly synchronized Jurkat T cells (21). We isolated RNA from Jurkat T cells...
and used a previously described reverse transcriptase PCR method (12) to amplify portions of the cDNAs encoding full-length iPLA2 protein and the alternatively spliced mRNAs predicted to encode the truncated proteins. As shown in Fig. 5B, this strategy amplified three products from the RNA of Jurkat cells at all stages of the cell cycle. The absolute intensities of these products varied from experiment to experiment, but their intensities relative to each other were consistent. The most abundant product migrated between the 220 and 298 kb markers and nucleotide sequencing (data not shown) indicated that it was derived from the mRNA encoding full-length iPLA2. Another of the PCR products migrated slightly faster than the 298 bp marker and nucleotide sequencing (data not shown) confirmed that it was the expected product from the ankyrin-iPLA2-1 mRNA (290 bp). The third product comigrated with the 344 bp marker and was predicted to be derived from the ankyrin-iPLA2-2 mRNA (predicted size = 342 bp). However, we were unable to clone this product and could not confirm its identity. The full-length iPLA2 mRNA was present throughout the cell cycle, and its levels appeared to increase during S phase. In contrast, the ankyrin iPLA2-1 mRNA was predominantly expressed during G2/M. To more accurately quantify the levels of full-length and ankyrin iPLA2-1 mRNAs, we performed real-time PCR using Taqman primers and probes designed to span the junction between exons 10 and exon 10a or exon 10 and exon 11 to specifically target the ankyrin-iPLA2-1 splice variant and full-length iPLA2 mRNAs, respectively. As shown in Fig. 5C, the relative amounts of the full-length and splice variant iPLA2 mRNAs varied during the cell cycle. The full-length iPLA2 was present during all phases of the cell cycle and exhibited a trend toward increased abundance in S phase that did not reach statistical significance when compared with the G2/M-arrested cells. In contrast, the splice variant iPLA2 mRNA was most abundant during G2/M (p < 0.05 compared with asyn, G1/S, and S phase cells).

We next performed experiments to determine if levels of the protein encoded by the splice variant would fluctuate during the cell cycle and be inversely correlated with iPLA2 activity. To test this hypothesis, we performed Western blot analysis on homogenates of synchronized Jurkat T cells using an anti-iPLA2 antibody (P-19) that recognizes an N-terminal epitope that is predicted to be present in both the full-length and truncated iPLA2 proteins. As shown in Fig. 6A, we detected a ~80 kDa band corresponding to full-length iPLA2, and the intensity of this band exhibited little change during the cell cycle. This observation was consistent with the results obtained with an antibody directed against the C terminus of iPLA2 in our CHO model (see inset, Fig. 4, A and B). In addition to the full-length iPLA2, the anti-N terminus antibody recognized a protein that was slightly larger than the 45 kDa molecular mass marker. This protein is approximately the expected size of the protein encoded by the Ankyrin-iPLA2-1 mRNA (53 kDa, predicted with the Peptide Sort program in the GCG package). Both of the immunoreactive bands were completely eliminated when the immunoblot was performed in the presence of the P-19 neutralization peptide (data not shown), indicating that the ~50 kDa protein was a truncated iPLA2 and not a nonspecific artifact of the immunoblot analysis. To our knowledge, this is the first demonstration of the proteins encoded by the iPLA2 splice variant mRNAs. Interestingly, the ratio of full-length iPLA2:truncated iPLA2 protein is highest during G2/M when iPLA2 activity is highest and lowest during G1/S and S phase when iPLA2 activity is lowest. Thus, the expression of truncated iPLA2 protein is cell cycle-dependent and inversely correlated with iPLA2 activity.

Having observed changes in the expression of ankyrin-iPLA2 during the cell cycle, we conducted several experiments to determine if this protein down-regulated iPLA2 activity through a physical association with full-length protein. To this end, we cloned hamster iPLA2 from CHO-K1 cells into the pTRE2Hyg inducible expression vector. Transient overexpression using this construct resulted in a marked increase in iPLA2 activity compared with empty vector (Fig. 7A). We then co-transfected the iPLA2 construct with the cDNA encoding human ankyrin-iPLA2-1. The expression of full-length iPLA2 was similar in cells transfected with full-length iPLA2 alone and cells transfected with full-length iPLA2 plus ankyrin-iPLA2-1 (Fig. 7A, inset). Unexpectedly, the anti-iPLA2 antibody (P-19) did not recognize the protein encoded by the ankyrin-iPLA2-1 cDNA (data not shown). However, our data suggest that this protein was expressed in the transfected cells, as iPLA2 activity was substantially lower in cells transfected with full-length CHO iPLA2 plus ankyrin-iPLA2-1 than in cells transfected with full-length CHO iPLA2 alone (Fig. 7A). This observation is consistent with that of Larsson et al. (12) who showed that ankyrin-iPLA2-1 suppressed the catalytic activity of human iPLA2. Together, these data support the hypothesis that ankyrin-iPLA2-1 is a negative regulator of CHO iPLA2 activity.

The ankyrin-iPLA2 proteins are proposed to act as negative regulators by binding to full-length iPLA2 and disrupting the formation of active tetramers (12). We conducted co-immunoprecipitation experiments to determine if full-length CHO iPLA2 interacts with ankyrin-iPLA2. A C-terminal-directed anti-iPLA2 antibody (T-14) was used to immunoprecipitate full-length iPLA2. This antibody recognizes an epitope that is present in full-length iPLA2 but not present in ankyrin-iPLA2-1. Proteins in the immunoprecipitates were separated by SDS-PAGE and probed with P-19, an antibody that recognizes an epitope that is present in both full-length iPLA2 and Ankyrin-iPLA2-1. Jurkat T cell extracts were used as a positive control for the migration of full-length and ankyrin-iPLA2 proteins (Fig. 7B, lane 1). The 80 kDa full-length iPLA2 was quantitatively immunoprecipitated from solution (lane 2) and cleared from the resulting supernatant (lane 3). The ankyrin-iPLA2 protein co-immunoprecipitated with full-length iPLA2 (lane 2). However, a fraction of the ankyrin-iPLA2 protein remained in the immunodepleted supernatant of the immunoprecipitation (lane 3) indicating that some of this protein is not associated with full-length iPLA2. The signals from full-length iPLA2 and the ankyrin-iPLA2 protein were absent when the immunoprecipitates were probed with P-19 antibody complexed to neutralizing peptide (Fig. 7B, Antibody + Peptide). Taken together, our results suggest that expression of the protein encoded by ankyrin-iPLA2-1 may regulate iPLA2 activity during the cell cycle and that the consequence of this regulation may be the net accumulation of PC during G1/S phase.

**DISCUSSION**

The objective of this study was to elucidate the mechanisms that allow for net accumulation of glycerophospholipids for cell division. Other investigators have shown that this process is largely mediated through reduced catabolism. Our data confirm and extend these observations by implicating iPLA2 as the major phospholipase regulated during the cell cycle. Furthermore, we show that iPLA2 activity declines during G1 and is lowest at the border of G1 and S phases, kinetics consistent with the accumulation of PC. Our data suggest that activity is down regulated through alternative splicing of the iPLA2 message, generating truncated proteins that accumulate during G1. Together, these data suggest that glycerophospholipid metabolism is regulated in the context of the cell cycle and iPLA2 activity.
FIG. 5. Cell cycle dependence of full-length iPLA$_2$ and ankyrin-iPLA$_2$ mRNA expression in Jurkat T cells. A, Jurkat T cells were arrested at different phases of the cell cycle as described under “Experimental Procedures.” A representative FACS analysis of DNA content is shown. Asyn (48 ± 7% G$_1$, 23 ± 6% S, 29 ± 12% G$_2$/M); G$_2$/M arrest (3 ± 2% G$_1$, 13 ± 7% S, 84 ± 7% G$_2$/M); G$_1$/S arrest (66 ± 9 G$_1$, 27 ± 5% S, 8 ± 5% G$_2$/M); Broad S Phase Arrest (12 ± 7% G$_1$, 32 ± 15% S, 12 ± 12% G$_2$/M); B, reverse transcriptase PCR was performed on total RNA from synchronized Jurkat T cells. The primers used recognize iPLA$_2$, iPLA$_2$-ankyrin-1, and iPLA$_2$-ankyrin-2. A representative experiment of four is shown. From left to right, DNA ladder, asyn cells, G$_2$/M cells, G$_1$/S cells, S phase cells. C, quantification of full-length and splice variant iPLA$_2$ mRNAs normalized to 18 S RNA (relative iPLA$_2$ expression). Primers and probes are described under “Experimental Procedures.” White bar, iPLA$_2$ mRNA; Gray bar, ankyrin-iPLA$_2$ mRNA. Data represent the mean ± S.E. of data compiled from three independent experiments. * indicates $p < 0.05$ versus G$_2$/M sample.
FIG. 6. Cell cycle dependence of full-length iPLA₂ and ankyrin-iPLA₂ protein expression in Jurkat T cells. A, equal amounts of protein from Jurkat T cells arrested at different phases of the cell cycle were subjected to Western analysis with the anti-iPLA₂ antibody (P-19) from Santa Cruz that recognizes an epitope common to the full-length and truncated iPLA₂ proteins. A representative blot (n = 4) is shown. The migration of the 83 kDa and 45 kDa protein standards is indicated. B, quantification of the ratio of full-length iPLA₂ to truncated iPLA₂ (ankyrin-iPLA₂) was performed through spot densitometry analysis using an Alpha Innotech Imaging system. Data represent the ratio of the integrated density values of full-length iPLA₂ over the integrated density values for the splice variant ankyrin-iPLA₂ for the experiment shown. Integrated density values for the 80 kDa iPLA₂ relative to the G/M sample: asyn = 1.3, G/M = 1.0, G/S = 1.3, S = 1.3. Integrated density values for the ankyrin-iPLA₂ relative to the G/M sample: asyn = 3.8, G/M = 1.0, G/S = 5.0, S = 5.8.

has a key role in this regulation.

It has long been established that glycerophospholipid metabolism is regulated during the cell cycle (13, 16, 18, 19). These studies have focused on synchronized populations of cells, typically generated through starvation followed by re-addition of growth factors or serum. Such cells typically exhibit a burst of PC synthesis when they reenter the cell cycle, but this does not correlate with the accumulation of PC mass that occurs during S phase. In fact, Terce et al. (13) suggest that the burst of PC synthesis is an adaptive response to the decline in PC mass that occurs in quiescent cells. We observed a ~7-fold increase in PC synthesis but a decline in PC mass when CHO cells were released from the thymidine/nocodazole block and allowed to progress from G₂/M into G₁ phase. These data suggest that the burst of PC synthesis may be a characteristic of G₁ phase cells and not simply an artifact associated with the transition from G₀ to G₁. Unfortunately as we could not maintain synchrony for a second cell cycle, we were unable to test this hypothesis by measuring the rate of PC synthesis when CHO cells transitioned from a second G₂/M phase to G₁ phase. However, our studies support the hypothesis that the rate of PC synthesis is highest during early G₁ and therefore that increased synthesis alone cannot account for the accumulation of glycerophospholipid mass during early S phase.

In most mammalian cells, the rate-limiting step in PC synthesis is catalyzed by CT. Although other investigators (16, 28) have shown significant (~2-10-fold) increases in CT activity when quiescent cells enter G₁, we were unable to observe any statistically significant increase in the in vitro catalytic activity of this enzyme. There are several potential explanations for this discrepancy. The source of enzyme in our CT activity assays was whole cell homogenates. Northwood et al. (28) were unable to measure increased CT activity in whole cell homogenates of IIC9 fibroblasts during G₁, but observed a modest increase (~2-fold) when activity was measured in digitonin-permeabilized “ghosts.” Thus, it is possible that the CT activity is induced in intact CHO cells, but that the activated form of CT is not preserved under our homogenization conditions. Using an immune complex assay, Jackowski (16) demonstrated a greater than 10-fold increase in CT activity when BACF1.2F5 macrophages made the transition from G₀ into G₁. However, the induction of CT activity was much more modest in subsequent cell cycles in which the cells transitioned from G₂/M to G₁ phase. The author attributed this to a loss of synchrony in the cell population. However, it is also possible that the dramatic increase in CT activity is a characteristic of cells making the transition from quiescence to G₁ and that induction is much more modest in cycling cells.

Using [³H]choline-labeled P815Y mast cells, Bergeron et al. (19) showed that the percentage of stably labeled lipids was highest during S phase, and thus were among the first to suggest that glycerophospholipid catabolism declines during S phase. In an elegant study, Jackowski directly measured the half-life of PC in a macrophage system (16) and showed that turnover was lower in S phase than in G₁ phase cells. Because of the nature of her system, Jackowski was unable to estimate PC turnover in G₂/M. With our model system, we have confirmed and extended these studies showing that the rate of PC turnover is 3-fold faster in G₂/M than it is in cells at the border of G₁ and S phases. These observations suggested that a phospholipase activity was down regulated during G₁.

Several lines of evidence suggest that iPLA₂ plays several key roles in the basal phospholipid metabolism of resting cells. For example, iPLA₂ activity is essential for the incorporation of arachidonic acid into the glycerophospholipids of P888D₁ macrophages (8). Studies in CHO, COS, and HeLa cells suggest that iPLA₂ activity and/or expression may be coordinately regulated with CTα to maintain PC mass (5, 6, 29). Although this does not appear to be the case in all cell types (10, 30), it suggests a role for iPLA₂ in glycerophospholipid homeostasis and we predicted that this enzyme would be regulated to allow for PC accumulation during S phase. Indeed, the kinetics of PC turnover correlated with the decline in iPLA₂ activity that we observed during G₁. iPLA₂ activity declined by ~55% during G₁ and reached its lowest level at the border of G₁ and S phases. These observations are consistent with a recent report showing ~70% less iPLA₂ activity in Jurkat T cells synchronized at G₂/S than in G₂/M cells (21). The decline in iPLA₂ activity not only
correlated with decreased turnover but was also consistent with the accumulation of PC mass during S phase. In contrast, cPLA2 activity did not change during the cell cycle. Although PLD activity exhibited a trend toward decreased activity in G1/S cells, this did not reach statistical significance and PLD activity exhibited a modest increase during S phase. Together, these data suggest that PLD is unlikely to contribute to the accumulation of phospholipid mass for cell division. However, as PLD activity has been shown to be essential for growth factor-induced mitogenesis (31–33), it remains possible that regulation of this enzyme contributes to lipid accumulation for daughter cell membranes in other cell systems. In addition, it is likely that PLD activity is essential for vesicular transport of the secretory pathway proteins synthesized during S phase (34). Together, our data suggest that down-regulation of iPLA2 activity may be essential for net glycerophospholipid accumulation in at least two very different cell types, CHO fibroblasts, and human T lymphocytes.

The most likely explanations for the decline in iPLA2 activity during G1 are a loss of protein mass or a decrease in the catalytic activity of the protein. Our data support the second hypothesis, as immunoblot and real time PCR analyses suggest that there is no decrease in the expression of full-length iPLA2 protein during G1 or S phase. To date, very little is known about the mechanisms that regulate iPLA2 activity. The enzyme requires ATP for maximal catalytic activity in vitro assays, but non-hydrolyzable ATP analogs will suffice suggesting that this is not mediated through phosphorylation (22). Larsson et al. (12) suggested a novel way to control iPLA2 activity, dependent on its presumed oligomerization into active homotetramers. This group reported that the iPLA2 gene undergoes alternative splicing, generating several splice variant mRNAs. The ankryin-iPLA2-1 and ankryin-iPLA2-2 splice variants are especially interesting for our studies as they are predicted to encode a premature stop codon that would generate truncated iPLA2 proteins containing the N-terminal ankyrin repeats but not the C-terminal catalytic site. Larsson et al. (12) demonstrated that the truncated proteins could suppress catalytic activity of human iPLA2, presumably by acting in a dominant negative manner to prevent the oligomerization of full-length iPLA2 proteins. We confirmed this result in CHO cells transfected with full-length hamster iPLA2 and human ankryin-iPLA2-1 cDNAs (Fig. 7A). To our knowledge, this is the first demonstration that human ankryin-iPLA2-1 can suppress
We were intrigued by the possibility that the truncated proteins might regulate iPLA2 activity during the cell cycle and used three strategies to test this hypothesis. In the first approach, iPLA2 cDNAs were amplified from Jurkat T cell RNA through a reverse transcriptase PCR using the primers reported by Larsson et al. (12). Three amplified products were observed and nucleotide sequencing confirmed that the smallest of these was derived from the mRNA encoding full-length iPLA2 protein and that the intermediate product was derived from an ankryin-iPLA2-splice variant. Importantly, the abundance of splice variant mRNA varied during the cell cycle and was highest during G2/M immediately prior to cell division. These observations were confirmed by quantitative real time PCR analyses. However, the iPLA2 splice variants appeared to be more abundant in real time PCR experiments than would be predicted based on the intensities of the products from the reverse transcriptase PCR. The real time PCR approach was designed to amplify any iPLA2 transcript containing the exon 10 - exon 10a border. To date, two such transcripts have been identified, ankryin-iPLA2-1 and ankryin-iPLA2-2 (12). However, it is possible that other such transcripts exist and that these novel transcripts do not hybridize to the primers used in our reverse transcriptase PCR experiments. Thus, the amplification of novel iPLA2 cDNAs may contribute to the signals obtained in the real time PCR experiments (Fig. 5C) but not to those from reverse transcriptase PCR (Fig. 5B) and explain the apparent discrepancy between the two approaches.

At first glance, the kinetics of the appearance of the splice variant mRNA in G2/M may appear to be inconsistent with down-regulation of iPLA2 activity during G1. However, G2/M is of short duration in our synchronization system as cells enter G1 within ~30 min of release from the nocodazole block (data not shown). Thus, we predict that alternative splicing of the iPLA2 pre-mRNA and transcription occurs during this short time frame and that the splice variant mRNAs are translated into protein when iPLA2 activity declines during G1.

Although the splice variant mRNAs have been demonstrated in human B cells, testes, and myeloid cells (35, 36), to date there is no conclusive evidence for the existence of the proteins encoded by these messages. Until recently, the only commercially available anti-iPLA2 antibodies were directed against the C terminus of the enzyme and therefore could not recognize the truncated proteins. However, Santa Cruz Biotechnologies has recently generated an antiserum directed against a peptide in the first 100 amino acids of iPLA2 that should recognize both full-length and truncated iPLA2 proteins.2 We used this antiserum to compare iPLA2 protein expression in Jurkat T cells at each stage of the cell cycle. In addition to recognizing a ~80 kDa protein (full-length iPLA2), the Santa Cruz antiserum detected a protein that was slightly larger than the 45 kDa molecular weight marker. The ~50 kDa protein was eliminated when the immunoblot was performed in the presence of a blocking peptide and was approximately the expected size of the proteins encoded by the splice variants (~53 kDa and ~55 kDa for ankryin-iPLA2-1 and ankryin-iPLA2-2 products, respectively). These data suggest that the ~50 kDa protein is derived from iPLA2 splice variant mRNAs and not a nonspecific artifact of the immunoblot analysis. To our knowledge, this is the first demonstration of a protein encoded by the iPLA2 splice variants. Importantly, the truncated protein accumulated with kinetics that were consistent with the cell cycle dependence of iPLA2 activity. Thus, the ratio of full-length to splice variant iPLA2 was lowest during late G1 and S phase when iPLA2 activity was low and was highest during G2/M when iPLA2 activity was high.

Based on these observations, we predict that the truncated protein associates with full-length iPLA2 and regulates iPLA2 activity during the cell cycle through the mechanism proposed by Larsson et al. (12, 35). Indeed, our co-immunoprecipitation

\[ \text{S. Barbour, personal communication.} \]
studies suggest that full-length iPLA2 and ankyrin-iPLA2 are physically associated during G2/M when iPLA2 activity is lowest. The co-immunoprecipitation experiment provides two additional insights into the biology of iPLA2 and its splice variants. The immunoprecipitates contained an additional immunoreactive protein that migrated slightly slower than ankyrin-iPLA2. This protein was absent when the blot was probed with neutralized antibody, suggesting that it is an iPLA2 protein and not a nonspecific artifact of the immunoblot. It is possible that this protein is encoded by yet another splice variant of the iPLA2 cDNA. We also observed a significant fraction of ankyrin iPLA2 protein in the supernatant of the immunoprecipitation. This observation suggests that some of the ankyrin iPLA2 protein is not associated with full-length iPLA2 in G2/S cells. Alternatively, it is possible that the interaction between full-length iPLA2, and the truncated protein is disrupted by our homogenization conditions.

Our results are summarized by the model depicted in Fig. 8. Our data indicate that splice variants of the iPLA2 mRNA are most abundant during G2/M. This suggests that the splicing of the iPLA2 message may be regulated in a cell-cycle-dependent manner. One potential mechanism for such regulation is through the SR proteins, a family of splicing factors whose subcellular localization and activity are proposed to be regulated by a cell cycle-regulated kinase, SRPK1 (37–40). Interestingly, a recent report suggests that SR proteins are dephosphorylated by a cell cycle-regulated kinase, SRPK1 (37–40). Unsaturated fatty acids activate the enzyme that generates ceramide that is induced by the bioactive lipid ceramide (41–43). Unsaturated fatty acids activate the enzyme that generates ceramide, neutral sphingomyelinase (44, 45), and one potential source of unsaturated fatty acids is iPLA2-mediated catabolism of glycerophospholipids. Thus, it is possible that fatty acid levels are elevated in G2/M due to high levels of iPLA2 activity and that these fatty acids activate sphingomyelinase-generating ceramide that promotes dephosphorylation of SR proteins and subsequent alternative splicing of the iPLA2 pre-mRNA. Our model predicts that the splice variant iPLA2 mRNA are translated into truncated proteins that accumulate during G2 and down-regulate iPLA2 activity by preventing the formation of active homotetramers. The decline in iPLA2 activity during G2 slows turnover and allows net accumulation to occur so that glycerophospholipids are available for cell division. However, once cells have entered S phase, iPLA2 activity begins to recover and reaches G2/M levels by the end of S phase. Our data indicate that splice variants of the iPLA2 mRNA are translated into truncated proteins that accumulate through interactions with Skp2. We are currently performing experiments to test the hypotheses prompted by this model and to more clearly elucidate the mechanisms that regulate iPLA2 activity during the cell cycle.

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