Effects of Protein Kinase CK2, Extracellular Signal-regulated Kinase 2, and Protein Phosphatase 2A on a Phosphatidic Acid-preferring Phospholipase A1*

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A soluble, phosphatidic acid-preferring phospholipase A1, expressed in mature bovine testes but not in newborn testes, may contribute to the formation of function of sperm. Here we incubated a recombinant preparation of the phospholipase in vitro with several enzymes including protein kinase CK2 (CK2), extracellular signal-regulated kinase 2 (ERK2), and protein phosphatase 2A (PP2A) to identify effects that might be of regulatory importance in vivo. Major findings were that 1) CK2 phosphorylated the phospholipase on serines 93, 105, and 716; 2) ERK2 phosphorylated the enzyme on serine 730; 3) there was cross-antagonism between the reactions that phosphorylated serines 716 and 730; 4) PP2A selectively hydrolyzed phosphate groups that were esterified to serines 716 and 730; 5) CK2α formed a stable, MgATP/MgGTP-dependent complex with the phospholipase by a novel mechanism; and 6) the complex showed reduced phospholipase activity and resembled a complex identified in homogenates of macaque testis. These results provide the first available information about the effects of reactions of phosphorylation and dephosphorylation on the behavior of the phospholipase, shed light on properties of CK2α that may be required for the formation of complexes with its substrates, and raise the possibility that a complex containing CK2α and the phospholipase may play a special biological role in the testis.

Mammalian tissues contain a soluble phospholipase A1 that can catalyze the preferential hydrolysis of PA in assays using mixed micelles (1) or unilamellar vesicles (2). The enzyme has been purified to homogeneity from bovine testes (3) and shown to have a molecular mass of 97.6 kDa, as determined by matrix-assisted laser desorption/ionization (4). Its cDNA has been cloned and sequenced and shown to encode an 875-amino acid protein that resembles other phospholipases only in so far as it contains a five-amino acid lipase consensus domain that includes a central serine residue (serine 540) required for catalysis (4). Moreover, analyses of the distribution of the human enzyme’s mRNA have provided evidence that this enzyme and one of its splice variants are expressed selectively in human tissues. However, the enzyme’s biological role remains to be determined, and little is known about the regulation of its activity inside cells.

The aim of the present investigation was to explore the possibility that protein kinases and phosphatases might affect the behavior of the first identified (bovine) splice variant of the enzyme, which we now call PA-PLA1α. We expressed an affinity-tagged, recombinant form of this enzyme in Sf9 cells, purified it, and examined the ability of several protein kinases and phosphatases to phosphorylate or dephosphorylate it in vitro. But only CK2 and ERK2 phosphorylated the phospholipase with significant stoichiometry, and only PP2A could catalyze the hydrolysis of the phosphatase esters. We used mass spectrometry to identify the amino acids that were phosphorylated or dephosphorylated and used several other approaches, including immunoprecipitation, quantitative densitometry, size exclusion chromatography, and enzyme activity analysis to characterize complexes of the enzyme that were formed in vitro or were identified in homogenates of the macaque testis and cerebral cortex.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides for expression of epitope-tagged PA-PLA1a in Sf9 cells (see below) were synthesized by Life Technologies, Inc. Phosphatidylcholine and sn-1-alkyl-2-oleoyl phosphatidic acid were purchased from Avanti Polar Lipids. FLAG peptide and FLAGM2 affinity beads (FLAGM2 antibody bound to Protein A-Sepharose beads) were from Sigma. [32P]ATP Easytide (specific activity 6000 Ci/mol) was from PerkinElmer Life Sciences. Recombinant forms of human CK2α, CK2β, λ protein phosphatase, and rabbit protein phosphatase 1 were from New England Biolabs. Constructs for bacterial expression of human GST-CK2α, GST-CK2β, and GST-CK2β were gifts from Dr. Dongxin Li. MAP kinase kinase-activated preparations of rat ERK2, rat c-Jun N-terminal kinase, mouse p38 MAP kinase, and starfish oocyte p34cdc2 kinase were from Calbiochem. MAP kinase-activated dotorpa fragipperda insect cells; Thesit, dodecylpoly(ethylene glycol)-S, where the average n is 9; PAGE, polyacrylamide gel electrophoresis.

J. Li, unpublished results.
ERK1 (rat) was from Upstate Biotechnology, Inc. (Lake Placid, NY). PPA2 (catalytic subunit), sequencing grade modified trypsin, and AspN were from Promega. Protein standards for size exclusion chromatography were from Bio-Rad. Sephadex G50, ATPγS, AMP-PNP, and the mixture of protease inhibitors used (“TM Complete Protease Inhibitor Mix, 1 mg/ml”; 100 units ml−1) were from Roche Applied Science. The peptide, pepstatin, phosphoramidon, pefabloc, EDTA, and aprotinin were from Roche Molecular Biochemicals. Thesil was from ICN Biochemicals. C18 microcolumn packing was from Michrom Bioreresources, Inc. High purity acetonitrile for high pressure liquid chromatography was from Burdick and Jackson. Glutathione-Sepharose 4B beads, Hi-Load Superdex 200 10/30 columns, and HiLoad Superdex 200 26/60 columns were from Amersham Pharmacia Biotech. PVDF membranes were from Millipore Corp. All other reagents were from Sigma or J.T. Baker Inc. unless mentioned otherwise.

Antibodies—Antibodies to CK2α, CK2α′, and CK2β, which had been prepared by David Litchfield, were gifts from Dr. Dongxia Li. The antibodies to ERK2 was from Calbiochem. Polyclonal antibodies against two peptides from PA-PLA1, TKKRIEELIRHLKSS (corresponding to a putative coiled-coil-forming region, Thr 589–Ser607) and KHEHDNNVKPSLDPV (corresponding to the C-terminal region, Lys881–Val897) were prepared by Research Genetics Inc. and subsequently affinity-purified on peptide columns, as described (4). Horseradish peroxidase-coupled anti-rabbit IgG antibody was from Amersham Pharmacia Biotech.

Expression and Purification of PA-PLA1α from SF9 Cells—The open reading frame of PA-PLA1α, attached at its 5′ end to a sequence of nucleotides that corresponded to the FLAG peptide (DYKDDDDK) followed by hexahistidine (HHHHHH), was cloned into pFASTBAC-HTc (corresponding to a putative coiled-coil-forming region, Thr 589–Ser607) and for the phosphorylated enzymes were separated by SDS-PAGE and stained with Coomassie Blue R-250. The gels were then dried and examined by autoradiography using Eastman Kodak Co. BIOMAX MS film. The identified bands were excised and soaked in 5 ml of Eulome (ICN Biochemicals). The radioactivity was measured with a Beckman scintillation counter, and the number of moles of phosphorus that had been used in the assays. This amount was determined by analysis using SDS-PAGE, Western blotting with the antibody to the coiled-coil-forming region, and quantitative densitometry (see below). Control incubation experiments using β casein or myelin basic protein as a substrate demonstrated that each of the kinases used in the above incubations was active.

Identification of Phosphorylated Sites by ESI-LC-MS/MS Analysis—After phosphorylating PA-PLA1α with CK2 or ERK2, we used SDS-PAGE to purify the 32P-labeled enzyme and then digested aliquots of the purified enzyme separately with trypsin and AspN as described by Lasky and co-workers (6). Briefly, the gel band containing the phosphorylated phospholipase was excised, dehydrated for 10 min in CH3CN, and dried in a Speedvac (Savant). Pieces of dried gel were incubated for 45 min at 4 °C in 100 μl of 50 μM NH4HCO3 that contained trypsin (12.5 μg/ml) or AspN (12.5 μg/ml). Then the temperature was increased to 37 °C, and the incubation was continued overnight. After the incubations, the digested PA-PLA1α was extracted from the gel pieces, first with the use of 100 μl of 20 mM NH4HCO3 and then with 100 μl of 50% CH3CN, 5% formic acid, 45% water. The extracted peptides were pooled and dried and then dissolved in solvent A, which contained 5% CH3CN, 0.4% acetic acid, 0.005% heptfluorobutyric acid in water. Approximately 200 fmol of the digested protein sample were loaded onto a homemade, 75-μm inner diameter microcolumn of C18, which had been prepared as described (7). Capillary LC was performed with the use of Applied Biosystems 1490 dual syringe pumps at a flow rate of 100 μl/min and a precolumn flow splitting ratio of 50:1, which resulted in a final flow rate through the column of 200 nl/min. After the sample was loaded, the column was washed with 5 μl with 100% solvent A. Then the peptides were eluted over a 60-min time period with a linear (0–80%) gradient of solvent B (80% CH3CN, 0.4% acetic acid, 0.005% heptfluorobutyric acid in water). The eluting peptides were analyzed by MALDI-TOF, MS/MS using a Finnigan LCQ ion trap mass spectrometer (Finnigan Mat LCQ, San Jose, CA) (8). ESI was performed using a needle voltage set at 1.8 kV. The heated capillary temperature was set at 170 °C. The scan range was 400–1800 m/z. The computer algorithm SEQUEST (9) was used to compare tandem mass spectra directly with amino acid sequence data bases and the PA-PLA1α sequence. Peptides that contained phosphorylated serine or threonine residues were identified by searching protein sequences that contained the serine or threonine residues corrected for the presence of phosphate ester groups (m +80). Results obtained from automated sequence data base searching were manually confirmed.

Analysis of Cross-antagonism between CK2α/β and ERK2—λ protein phosphatase-pretreated PA-PLA1α (0.5 μg) was incubated for 30 min at 30 °C with CK2α/β (500 units) in 25 μl of phospholipase buffer that contained unlabeled MgATP (0.55 mM). Then ERK2 (120 units) and [32P]ATP (100 μM) were added in 10 μl of phospholipase buffer and the incubation was continued for an additional 60 min. Alternatively, the phospholipase was incubated for 30 min with ERK2 plus unlabeled ATP. Then CK2α/β and radioactive ATP were added, and the incubation was continued for an additional 60 min. In either case, the reaction was stopped by the addition of SDS sample buffer, the mixture was boiled for 5 min, radioactive PA-PLA1α was isolated by SDS-PAGE, the gel was stained with Coomassie Blue R-250, the band of radioactive phospholipase was excised and counted in a scintillation counter, and the number of moles of phosphorus that had been incorporated per mole of phospholipase in the incubations with radioactive ATP was calculated on the basis of the combined specific radioactive of ATP in the incubations.

Dephosphorylation of PA-PLA1α by PPA2—PA-PLA1α that had been phosphorylated by either CK2 or ERK2, as mentioned above, was brought up to a volume of 100 μl by the addition of 50 mM Tris-HCl, pH 8.5, 20 mM MgCl2, 1 mM DTT, 0.01% β-mercaptoethanol, 0.1 mM/BSA; passed through a column of Sephadex G50 to remove free nucleotide.
PA-PLA1α Phosphorylation and Diphosphorylation

otides; and incubated for 30 min at 30°C with PP2A. The reaction mixture was then boiled in SDS sample buffer, PA-PLA1α from the reaction mixture was purified by SDS-PAGE, and the amount of radioactive phosphate that remained associated with the phosphoprotein was calculated, as described above.

Binding of PA-PLA1α to CK2αββ2 or Its Subunits—Recombinant, GST-tagged preparations of CK2 αββ2 (4 µg), CK2 eββ2 (4 µg), CK2α (2 µg), CK2α′ (2 µg), or CK2β (2 µg) were bound separately to beads of glutathione-Sepharose 4B and then incubated for 30 min at 30°C with diphosphorylated recombinant PA-PLA1α (5 µg) in 500 µl of phosphoprotein buffer. After the incubations, the beads were washed three times with 1 ml of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and then extracted with SDS sample buffer. The PA-PLA1α in the extracts was purified by SDS-PAGE and transferred to PVDF membranes; then the enzyme protein on the membranes was identified by Western blotting using the antibody to the putative coiled-coil-forming region of PA-PLA1α.

Relation between Phosphorylation of PA-PLA1α by CK2α and Complex Formation by the Two Enzymes—α protein phosphatase-pre-treated, recombinant PA-PLA1α (0.2 pmol) was incubated for 30 min at 4°C with FLAGM2 affinity beads (40 µl of a 50% slurry) in homogenization buffer (500 µl) that contained 0.1 mM Thesit. After the incubation, aliquots of the beads were washed three times with 1 ml portions of homogenization buffer (to remove unbound PA-PLA1α) and then incubated for 120 min periods with 50 µl of recombinant CK2α in phosphorylation buffer (25 µl) containing 32P-labeled ATP (for phosphorylation studies) or unlabeled MgATP (for studies of complex formation). After each incubation, the beads were washed three times with 1 ml of homogenization buffer, the reactions were stopped by the addition of SDS sample buffer, the mixtures were boiled for 5 min, and the enzymes they contained were purified by SDS-PAGE and stained with Coomassie Blue R-250. After this, phosphoprotein incorporation into the enzymes was measured as described under “Measurements of Phosphorylation Reaction Stoichiometry after Incubations Involving Soluble Enzymes,” or the time course and stoichiometry of complex formation by PA-PLA1α and CK2α was determined by transferring the enzymes to PVDF membranes and probing them with the antibody to CK2α or the coiled-coil-forming region of PA-PLA1α followed by the horseradish peroxidase-coupled antibody to rabbit IgG. The response of each enzyme was visualized by enhanced chemiluminescence (2), quantitated with the use of a Bio-Rad model GS-700 imaging densitometer (2), and converted into a molar concentration by comparison with signals from standards containing recombinant GST-CK2α. Different amounts of sample were analyzed to ensure that the amount of GST-CK2α measured fell within the linear range of the standard curve.

Stability of the Complex between PA-PLA1α and CK2α—PA-PLA1α that had been immobilized on FLAGM2 beads was incubated for 30 min at 30°C with CK2α and MgATP in phosphorylation buffer. Then the beads were washed with homogenization buffer to remove unbound CK2α and either directly with SDS sample buffer or incubated separately for 30 min at 30°C in control buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, or 350 mM KCl) plus 1 mM DTT (a mixture of ingredients reported to support the metabolism of permeabilized mammalian cells (10)) and 2) the following mixture of protease inhibitors: 1 µM benzamidine, 1 mM phenylmethanesulfonyl fluoride, 2 mM each of leupeptin, pepstatin, and aprotinin. A similar buffer was used for the size exclusion chromatography at a flow rate of 0.4 ml/min. An aliquot (50 µl) of each fraction (0.5 ml) from the column was boiled on an Immobilon-P PVDF membrane (Millipore) using a Bio-Dot apparatus (Bio-Rad) and probed with either an antibody to the predicted coiled-coil-forming region of PA-PLA1α or as described above or an antibody to CK2α. The chemiluminescent response also was measured, as described (2). Then molecular masses of the analyzed proteins and complex were determined on the basis of comparisons of their elution volumes with those of the Bio-Rad size exclusion standards, blue dextran 2000 (2000 kDa), bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), chicken ovalbumin (44 kDa), horse myoglobin (17 kDa), and vitamin B12 (1.3 kDa).

Analysis of Complexes Containing PA-PLA1α in Homogenates of Macaque Testis or Cerebral Cortex—Testes or brain frontal cortical regions were removed from adult male macaques shortly before death and minced by hand. The minced tissues were washed three times with ice-cold cytosolic buffer and then homogenized in 3 volumes of this buffer using a Potter-Evvelhej homogenizer. The homogenate was centrifuged for 10 min at 800 x g, and the low speed supernatant was collected and centrifuged for 1 h at 235,000 x g in a Beckman Ti 50 rotor. The resulting high speed supernatant was flash-frozen in 10-ml aliquots. Subsequently, the aliquots were thawed separately and loaded at a flow rate of 2.6 ml/min onto a column of HighLoad Superdex 200 (26/60) that had been pre-equilibrated with cytosolic buffer as described above for the size exclusion chromatography of recombinant proteins. Fractions were collected at 1.8-ml intervals beginning at 107 ml. Aliquots of the fractions were analyzed by Western blotting and quantitative densitometry using antibody to the C-terminal region of PA-PLA1α, and peaks containing the enzyme were pooled and concentrated to a final volume of about 1 ml using a Centrinic concentrator (Millipore).

Finally, the concentrated peaks containing PA-PLA1α from the testes or brains were precleared by treatment for 1 h at 4°C with 2 µg of affinity-purified affinity-purified rabbit IgG against recombinant human PA-PLA1α (50% slurry). The remaining, unabsorbed material was incubated for 3 h at 4°C with antibody to the C-terminal region of PA-PLA1α (2 µg) plus protein A-Sepharose beads (40 µl). The beads were then washed three times with 1 ml of cytosolic buffer, extracted with SDS sample buffer, boiled for 5 min, and analyzed by Western blotting using antibodies to PA-PLA1α, CK2α, or CK2β.

Analysis of Complex Formation between PA-PLA1α and ERK2—Recombinant PA-PLA1α (0.5 µg) that had been pretreated with λ protein phosphatase was immobilized on FLAGM2 beads and then incubated for 30 min at 30°C with recombinant ERK2 (2 µg) in 500 µl of phosphorylation buffer in the presence or absence of 0.55 mM MgATP. After the incubations, the beads were washed three times with 1 ml portions of 50 mM Tris-HCl, pH 7.5, plus 150 mM NaCl and then extracted with SDS sample buffer. The extract was analyzed by SDS-PAGE and transferred to PVDF membranes. Then an antibody to ERK2 was used to probe the membranes.

Other Methods—Proteins were measured by the micro-BCA method (Bio-Rad). Phosphorylation sites were predicted with the use of the protein data base search software program PROSITE (available on the World Wide Web) for protein functional regions and post-translational modifications. The molecular masses of recombinant, epitope-tagged PA-PLA1α and GST-CK2α were determined with the software program PeptideMass (available on the World Wide Web). Statistical analyses were done with Microsoft Excel. Molecular modeling studies of the predicted coiled-coil-forming region were done with Rasmol.

RESULTS

CK2αββ2, CK2α, CK2α′, and a Putative S9 Cell MAP Kinase Phosphatase Phosphatase PA-PLA1α—The sequence of PA-PLA1α contains predicted phosphorylation sites for several protein kinases including CK2αββ2 (not shown). To investigate the possibility that CK2αββ2 might catalyze the phosphorylation of the
phospholipase in vitro, we incubated purified recombinant preparations of the two enzymes together for 60 min in the presence of radioactive ATP and then measured the stoichiometry of PA-PLA1α phosphorylation (see “Experimental Procedures”). The results of six experiments demonstrated that 2 mol of phosphorus were incorporated per mole of the phospholipase (Table I). To identify the sites that were phosphorylated, we analyzed digests of the phospholipase by ESI-LC-MS/MS (see “Experimental Procedures”). Unexpectedly, the analysis identified four phosphorylated peptides, not two, as would have been predicted from the measurements of phosphorylation stoichiometry that were made after the in vitro incubations. Moreover, each peptide had a molecular mass that exceeded the value predicted from cDNA sequence analysis by m + 80 and therefore contained a single esterified phosphate group.

Three of the peptides contained serine residues that preceded nearby glutamates and were predicted CK2αβ2 phosphotyrosine sites. 1) Peptide 913D...R1022 was phosphorylated on serine 93 (Fig. 1A). 2) Peptide 1023Y...R1413, which contained eight serines, appeared to be phosphorylated only on serine 105 (Fig. 1B). We identified this phosphorylation site by exclusion on the basis of the combined results of the b and y ion series. We detected a mass of +80 in the b ion series b14... (1023...S105) but were unable to assign the phosphate from this series. On the other hand, we detected no mass of +80 in y1–37 (1413R...G105), which contained seven out of the eight serines in the sequence, serines 109, 114, 115, 116, 117, 128, and 130. 3) Peptide 713D...R735 was phosphorylated on serine 716 because there was a loss of mass of 80 between b9 and b10 (Ile715 and Glu717; Fig. 1C).

The fourth phosphorylated peptide, 786D...L812, contained an esterified phosphate group on serine 793, which was not a predicted CK2αβ2 phosphotyrosine site (Fig. 1D). Instead, proline residues were present nearby, consistent with the possibility that serine 793 might have been phosphorylated by a proline-directed kinase in Sf9 cells. Note that others have shown that Sf9 cells contain a MAP kinase that can phosphorylate a recombinant form of the arachidonoyl-specific phospholipase A2 (11) but also that the phosphorylated peptide (786D...L812), which we detected, comprised only a minority of the total 786D...L812 peptide identified in the digest of recombinant PA-PLA1α.

The results of other experiments using a preparation of the phospholipase that had been treated with λ protein phosphatase before being incubated with radioactive ATP plus either CK2αβ2 or CK2α or CK2αβ2 showed that each of the three preparations of CK2 could catalyze the incorporation of a maximum of 3 mol of phosphorus into the phospholipase (Table I; also see Fig. 5A). Therefore, it appeared that all three forms of CK2 could phosphorylate the phosphatase-pretreated PA-PLA1α on serine 93, serine 105, and serine 716.

The fact that incubation with CK2 in vitro caused two phosphate groups to be incorporated into the untreated phospholipase but three to be incorporated into the treated phospholipase suggested that Sf9 cells might have partially phosphorylated the phospholipase on CK2-dependent sites during its expression. In support of this possibility, the mobility of a preparation of untreated phospholipase that had not been incubated with CK2 in vitro increased when it was incubated with λ protein phosphatase, as determined by SDS-PAGE (data not shown), and analysis of the resulting product by mass spectrometry provided evidence that it contained no residual esterified phosphate (data not shown). However, we were unable to obtain further proof that Sf9 cells had phosphorylated the enzyme on CK2-dependent sites by examination of a digest of a phospholipase preparation that had been exposed to neither the kinase nor the phosphatase. The only phosphorylated peptide that we could detect was one containing serine 793 (data not shown). The MS analysis was not conclusive, so we may well have missed peptides that were phosphorylated on CK2 sites in low stoichiometry. But conclusive proof that Sf9 cells phosphorylated the phospholipase on CK2 sites remains to be obtained.

**ERK2 Phosphorylates PA-PLA1α**—In subsequent in vitro incubation experiments, we attempted to identify a MAP kinase that could catalyze the phosphorylation of serine 793, as the putative proline-directed kinase in Sf9 cells did. We incubated λ phosphatase-pretreated, recombinant PA-PLA1α with radioactive ATP plus a constitutively activated form of either ERK1, ERK2, c-Jun N-terminal kinase 1, p38, or p34cdc2 but found that only ERK2 could phosphorylate the PA-PLA1α with significant stoichiometry. It catalyzed the incorporation of 1 mol of phosphate/mol of the phospholipase (Table II), and we identified only one phosphorylated peptide, 713D...R735 upon examining digests of the ERK2-phosphorylated phospholipase using LC-MS/MS (Fig. 2). Identification of the phosphorylated amino acid in this peptide was difficult at first because of the peptide’s fragmentation pattern and its content of four prolines, six serines, and three threonines. But we scanned through a narrow window of m/z 2500–3000 using alternate MS-MS/MS in a second run and were able to identify serine 730 as the only phosphorylated residue. Thus, ERK2 phosphorylated the enzyme in vitro but on a different site than the putative proline-directed protein kinase in Sf9 cells did. Therefore, the identity of this Sf9 cell protein kinase remains to be determined.

**Selective, Cross-antagonism of Phosphorylation by CK2 and ERK2**—The proximity of the CK2 phosphorylation site, serine 716, and the ERK2 phosphorylation site, serine 730, raised the possibility that phosphorylation reactions involving the two sites might influence each other. To examine this possibility, we first incubated a λ phosphatase-pretreated preparation of recombinant PA-PLA1α for 30 min at 30 °C with CK2αβ2 plus unlabeled ATP under conditions that could cause the incorporation of 3 mol of phosphate into each mole of the phospholipase and then added ERK2 plus radioactive ATP to the mixture and continued the incubation for an additional 60 min. The results revealed that less than 0.1 mol of radioactive phosphate became incorporated into the phospholipase during the incubation with ERK2 (Table II). Moreover, when we incubated recombinant PA-PLA1α with ERK2 plus unlabeled ATP in a parallel experiment and then added CK2αβ2 plus radioactive ATP and continued the incubation, 2 mol of radioactive phosphate, not 3 mol, were incorporated per mole of the phospholipase. Because incubation experiments with mixtures of the phospholipase plus radioactive ATP plus either ERK2 or CK2αβ2 without pretreatment with the other kinase showed significantly higher amounts of incorporated radioactive phosphate (Table II), these results provided evidence that phospho-

| TABLE I Stoichiometry of PA-PLA1α phosphorylation by CK2 |
|-----------------------------------------------------------|
| Recombinant PA-PLA1α that had or had not been pretreated with λ protein phosphatase was incubated for 60 min at 30 °C with recombinant preparations of CK2αβ2, CK2α, or CK2αβ2 in the presence of radioactive ATP, and the stoichiometry of phosphorylation was measured (see “Experimental Procedures”). Values are means ± S.E. from duplicate analysis of six experiments for the nonpretreated PA-PLA1α sample and three experiments for the pretreated PA-PLA1α sample. |
| PA-PLA1α preparation | Kinase | Mol of P/mol of PA-PLA1α |
|----------------------|-------|--------------------------|
| Nonpretreated        | CK2αβ2 | 2.1 ± 0.27               |
| Pretreated           | CK2αβ2 | 2.9 ± 0.14               |
| Pretreated           | CK2α  | 2.9 ± 0.13               |
| Pretreated           | CK2αβ2 | 2.9 ± 0.14               |
| a Plateau values that differ significantly (Student’s t test) at p < 0.01. |
rylation of the phospholipase by CK2 inhibited the subsequent phosphorylation of the phospholipase by ERK2 and vice versa. Furthermore, analysis by LC/MS/MS showed that the inhibitory effect of phosphorylation by ERK2 on phosphorylation by CK2 specifically involved the CK2 phosphorylation site, serine 716. Thus, upon phosphorylating the enzyme successively with ERK2 and CK2, we could identify only three phosphorylated amino acid residues: the CK2 phosphorylation sites, serine 93 and serine 105, and the ERK2 phosphorylation site, serine 730 (scans not shown).

FIG. 1. Identification of phosphorylated sites after incubation of recombinant PA-PLA1α with CK2αβ2 and MgATP. Recombinant PA-PLA1α (which had not been pretreated with λ protein phosphatase) was incubated for 30 min at 30 °C with CK2αβ2 plus [γ-32P]ATP and then purified by SDS-PAGE, subjected to in-gel digestion with trypsin or AspN, and analyzed by micro-LC/MS/MS (see "Experimental Procedures"). A–C, CK2 phosphorylation sites; D, presumptive proline-directed protein kinase phosphorylation site. Sequences of identified phosphopeptides are shown above the mass spectra; phosphorylation sites are indicated by asterisks; numbers in superscript on the amino and carboxyl termini of each peptide denote the location of the peptide within the PA-PLA1α sequence; b, denotes the ion generated by cleavage of the peptide bond after the nth amino acid from the amino terminus; γ, denotes the ions generated from the carboxyl terminus; identified b or y ions are shown in boldface letters; and values of m/z (mass/charge) for ions are indicated in the mass spectra.

PP2A Dephosphorylates PA-PLA1α Selectively—To identify a physiologically relevant protein phosphatase that could dephosphorylate the phospholipase by ERK2 and vice versa. Furthermore, analysis by LC/MS/MS showed that the inhibitory effect of phosphorylation by ERK2 on phosphorylation by CK2αβ2 specifically involved the CK2αβ2 phosphorylation site, serine 716. Thus, upon phosphorylating the enzyme successively with ERK2 and CK2αβ2, we could identify only three phosphorylated amino acid residues: the CK2αβ2 phosphorylation sites, serine 93 and serine 105, and the ERK2 phosphorylation site, serine 730 (scans not shown).

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were not subsequently treated with PP2A. After the incubations, radioactive phosphorus (P) incorporated into the phospholipase was determined on the basis of the combined specific radioactivity of MgATP in the incubations (see “Experimental Procedures”). The sequence of the phosphopeptide, the phosphorylation site, the identified b or y ions, and the m/z value are shown as in Fig. 1.

| Kinase | Mol of radioactive P/mol of PA-PLA1α |
|-------|-------------------------------------|
| ERK2 only | 1.01 ± 0.12^a |
| CK2αββ, ERK2 | 0.10 ± 0.01^a |
| CK2αββ, only | 2.90 ± 0.14^a |
| ERK2, CK2αββ | 1.80 ± 0.10^a |

^a Significantly different (Student’s t test) at p < 0.02.

**TABLE III**

**PP2A dephosphorylates PA-PLA1α selectively**

Samples containing λ protein phosphatase-pre-treated recombinant PA-PLA1α were incubated for 30 min at 30 °C in the presence of radioactive 32P-labeled ATP plus either CK2αββ or ERK2 and then treated for 30 min with PP2A at 30 °C (see “Experimental Procedures”). Alternatively, control samples of the phospholipase were incubated for 30 min with radioactive 32P-labeled ATP and CK2αββ or ERK2 but were not subsequently treated with PP2A. After the incubations, radioactive phosphorus (P) in PA-PLA1α was measured as described (see “Experimental Procedures”). Results represent mean ± S.E. of duplicate measurements from two different sets of experiments.

| Kinase | Mol of radioactive P/mol of PA-PLA1α |
|-------|-------------------------------------|
| CK2αββ | 2.90 ± 0.14^a |
| CK2αββ, PP2A | 1.80 ± 0.12^a |
| ERK2 | 1.03 ± 0.16^a |
| ERK2, PP2A | 0.10 ± 0.10^a |

^a Significantly different (Student’s t test) at p < 0.01.

CK2α or CK2αββ dissociate when they are treated with 350 mM KCl (Table IV and data not shown).

To determine whether PP2A catalyzed the complete removal of phosphate from a single site on the phospholipase or catalyzed the partial removal of phosphates from several sites, we used LC-MS/MS to analyze digests of phospholipase that had been successively phosphorylated by CK2αββ and then dephosphorylated by PP2A. The results of the analysis demonstrated that serine 716 had lost 80 mass units, whereas serine 93 and 105 were fully phosphorylated (Fig. 3 and data not shown). Therefore, the action of the phosphatase was site-specific.

When we did a comparable set of incubation experiments using PA-PLA1α that had been phosphorylated by ERK2, we found that PP2A could hydrolyze almost all of the phosphate that was esterified to the phospholipase, whereas both protein phosphatase 1 and calcineurin were without effect (Table III and data not shown). Since ERK2 could phosphorylate only one site, serine 730, this site was clearly the one that was dephosphorylated. Therefore, PP2A dephosphorylated both serine 716 and serine 730, the same two serines that showed cross-antagonism of phosphorylation by CK2αββ and ERK2. Whether this was coincidental or reflected a special structural feature of the PA-PLA1α remains to be determined (but see “Discussion”).

**TABLE IV**

**Effects of Triton X-100, PP2A, and KCl on the stability of the PA-PLA1α plus CK2α complex**

PA-PLA1α that had been immobilized on FLAG beads was incubated for 30 min at 30 °C with CK2α plus MgATP in phosphorylation buffer (see “Experimental Procedures”). Samples containing the immobilized complex of PA-PLA1α plus CK2α were then washed with homogenization buffer to remove unbound CK2α and subsequently incubated separately for 30 min at 30 °C in buffer containing 50 mM Tris-HCl, pH 8.5, 20 mM MgCl2, 1 mM DTT, 0.1% β-mercaptoethanol, 0.1 mg/ml BSA 1% (control) or buffer plus either one of the following components: 1% Triton X-100, 150 mM KCl, PP2A, or 350 mM KCl. At the end of incubation, the amount of CK2α in the supernatant was analyzed by SDS-PAGE, Western blotting, and quantitative densitometry. The total CK2α present in the original complex was determined by extraction of CK2α from the complex with SDS sample buffer and analyzing the CK2α in the extract using a similar approach (data not shown). The results indicate means ± S.E. from two different experiments.

| Samples in buffer | Relative densitometric units × 1000 | Percentage of removal |
|-------------------|------------------------------------|-----------------------|
| Complex (control) | 11.3 ± 0.003 | 3.5 |
| Complex + 1% Triton X-100 | 52.0 ± 0.006 | 16.3 |
| Complex + 150 mM KCl | 37.8 ± 0.007 | 11.8 |
| Complex + PP2A | 24.6 ± 0.001 | 7.7^a |
| Complex + 350 mM KCl | 286 ± 0.01 | 89.6 |

^a 39.3 and 32.8% of radioactivity (about one phosphate) was removed when the complex containing phosphorylated PA-PLA1α plus CK2α complex (in the presence of [32P]ATP) was dephosphorylated with PP2A (data not shown).
provided evidence that the phosphorylation of PA-PLA1α was promoted by CK2α and that it was relatively resistant to extraction with 1% Triton X-100 or 150 mM KCl and remained essentially intact after approximately one-third of the phosphorus esterified to PA-PLA1α was removed by treatment with PP2A (Table IV). This indicated that the stability of the complex did not depend on the PP2A-sensitive phosphate group that was esterified to serine 716 (see Table III and Fig. 3). Importantly, however, the complex dissociated when it was treated with 350 mM KCl (Table IV), which suggested that the stability of the complex depended on electrostatic interactions involving other components of the two enzymes.

**Molecular Basis of Complex Formation**—To examine the possibility that these electrostatic interactions might have involved the phosphate groups that were esterified to serines 93 and 105, we took advantage of the results of the above experiments to prepare phosphorylated, kinase-free forms of the phospholipase that contained esterified phosphate groups on serines 93 and 105, we took advantage of the results of the above experiments to prepare phosphorylated, kinase-free forms of the phospholipase that contained esterified phosphate groups on serines 93 and 105, or serines 93, 105, and 716 (see “Experimental Procedures”). Upon incubating these preparations separately with fresh CK2α, we were surprised to find that even the maximally phosphorylated form of PA-PLA1α did not bind the CK2α except in the presence of MgATP (Fig. 6A). Furthermore, MgGTP could substitute for the MgATP, but MgADP, MgUTP, and MgCTP were without effect (Fig. 6, A and B). These results left open the possibility that the formation of the complex might have depended on the MgATP-dependent phosphorylation of

In further studies, we focused attention on the complex that contained PA-PLA1α and CK2α. To examine the relation between the phosphorylation of the phospholipase by CK2α and the formation of this complex, we incubated the two enzymes together for periods of 5–120 min in the presence of unlabeled MgATP or radioactive ATP and compared the time courses and reaction stoichiometries of these processes (see “Experimental Procedures”). The results of two independent experiments demonstrated that 1) the time courses of PA-PLA1α phosphorylation and complex formation were similar, although not completely identical, 2) 3 mol of phosphorus were ultimately incorporated into each 97.6-kDa molecule of PA-PLA1α, 3) the complex ultimately contained 0.8 mol of CK2α/mol PA-PLA1α, and 4) no detectable radioactive phosphate was incorporated into CK2α (Fig. 5, A and B). This provided evidence that the phosphorylation of PA-PLA1α by CK2α promoted the formation of a stable, 1:1 complex between the two enzymes.

We next examined the stability of the complex and found that it was relatively resistant to extraction with 1% Triton X-100 or 150 mM KCl and remained essentially intact after approximately one-third of the phosphorus esterified to PA-PLA1α was removed by treatment with PP2A (Table IV). This indicated that the stability of the complex did not depend on the PP2A-sensitive phosphate group that was esterified to serine 716 (see Table III and Fig. 3). Importantly, however, the complex dissociated when it was treated with 350 mM KCl (Table IV), which suggested that the stability of the complex depended on electrostatic interactions involving other components of the two enzymes.

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FIG. 3. PA-PLA1α that has been phosphorylated by CK2αβ can be selectively dephosphorylated by PP2A. PA-PLA1α that had been pretreated with λ protein phosphatase was incubated for 30 min at 30 °C with CK2αβ and MgATP and then isolated by size exclusion chromatography and incubated with PP2A as described under “Experimental Procedures.” Afterward, the PA-PLA1α was purified by SDS-PAGE, and peptides obtained by in-gel digestion were analyzed by LC/MS/MS (see “Experimental Procedures”). The sequences of the phosphorus-containing peptides, identified by b or y ions, and m/z values are shown as in Fig. 1.

FIG. 4. PA-PLA1α forms stable complexes with CK2. Samples of GST-linked CK2αβ, CK2α, CK2β, or GST alone, which had been immobilized on glutathione-Sepharose 4B beads, were incubated separately with PA-PLA1α plus MgATP for 30 min at 30 °C. The beads were washed with homogenization buffer, extracted with SDS sample buffer, and analyzed by SDS-PAGE followed by Western blotting with the antibody to the coiled-coil-forming region of PA-PLA1α. Similar results were obtained in two additional experiments.

FIG. 5. Relation between the phosphorylation of PA-PLA1α by CK2α and the formation of a complex between the two enzymes. PA-PLA1α that had been immobilized on FLAGM2 affinity beads was incubated with GST-CK2α for periods of 5–120 min in phosphorylation buffer that contained 32P-labeled ATP or unlabeled MgATP. After each incubation period, the beads were washed with homogenization buffer and extracted with SDS-sample buffer. Then the two enzymes were purified by SDS-PAGE, and the time courses of PA-PLA1α phosphorylation (A) and complex formation (B) were determined as described under “Experimental Procedures.” Note that no incorporation of radioactive phosphorus into CK2α was detected, that the results represent duplicate analyses from two different experiments, and that similar results were obtained when time course experiments were performed with enzymes in solution.
serines 93 and 105 but showed that an additional, highly specific, MgATP/MgGTP-dependent mechanism was involved. Because CK2α can accommodate either MgATP or MgGTP in its Mg-trinucleotide-substrate-binding site (14) and could phosphorylate PA-PLA1α in the presence of either one (data not shown), it seemed likely that MgATP or MgGTP contributed to the formation of the complex by binding to this site (see “Discussion”).

**Evidence That the Complex of PA-PLA1α and CK2α Contains Four Molecules of Each Protein**—We used size exclusion chromatography to estimate the molecular masses of soluble preparations of recombinant PA-PLA1α and GST-tagged CK2α and to compare them with that of a complex of the two enzymes that had been formed in the presence of MgATP (Fig. 7). When the recombinant PA-PLA1α was chromatographed by itself, it had an apparent molecular mass of 220 kDa, which provided evidence that it was a dimer. On the other hand, the GST-tagged CK2α was a monomer because it had a molecular mass of 62 kDa, as compared with the expected mass of 68 kDa (26-kDa GST plus 42-kDa CK2α). In view of these results, we expected that a 1:1 complex of the two enzymes that were bound to PA-PLA1α would have an apparent molecular mass of about 340 kDa. However, upon incubating the enzymes together under the appropriate conditions and examining the resulting complex by size exclusion chromatography, we found that the complex had an apparent molecular mass of about 650 kDa, which suggested that it was a heterooctamer formed from four molecules of PA-PLA1α and four molecules of GST-tagged CK2α. These results suggested that at least three types of binding sites contributed to the complex: 1) a binding site on the phospholipase that promotes the formation of an enzyme dimer, 2) a binding site for CK2α on each subunit of the dimer, and 3) a binding site that promotes the conversion of a presumptive, intermediate enzyme heterotetramer into a heterooctamer. Further structural work will be required to clarify the molecular basis of these binding phenomena.

**The Effects of Phosphorylation and Complex Formation on the Phospholipase Activity of PA-PLA1α**—To examine the effect of phosphorylation by CK2α on the catalytic properties of PA-PLA1α, we incubated the kinase with the λ protein phosphatase-pretreated phospholipase in the presence of unlabeled MgATP under conditions that could cause complex formation (control experiments using radioactive ATP confirmed that 3 mol of phosphorus were incorporated into the phospholipase) and then used a mixed micelle assay or a unilamellar vesicle assay to measure the activity of the phospholipase (see “Experimental Procedures”). The results of two independent experiments with each type of assay indicated that the incubation caused a 50% loss of phospholipase activity (Table V). These results differed from those of corresponding incubation experiments with PA-PLA1α plus ERK2 and MgATP (see “Experimental Procedures”), which provided no evidence for complex formation or phosphorylation-dependent loss of phospholipase activity (data not shown and Table V).

**Macaque Testis Contains a Complex of CK2α and PA-PLA1α, but Macaque Cerebral Cortex Does Not**—After characterizing the complex that PA-PLA1α formed with CK2α in vitro, we investigated the possibility that homogenates of freshly obtained macaque testes might contain a similar complex. We centrifuged the homogenates to prepare a high speed supernatant fraction from each one and then analyzed aliquots of this fraction by size exclusion chromatography (see “Experimental Procedures”). Immunoblot analysis of the effluent from the chromatography columns using the C-terminal antibody to PA-PLA1α revealed the presence of cross-reactive material in both the void volume peak and a complex, second peak that emerged immediately afterward (Fig. 8A). Note that the molecular mass of the material in each peak was equal to or greater than 670 kDa. To analyze the material in the peaks in more detail, we pooled the fractions that comprised each peak, concentrated the pools, and then used the antibody to

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**Fig. 6.** Specificity of the nucleotide requirement for complex formation between PA-PLA1α and CK2α. Aliquots of GST-CK2α were incubated separately for 30 min at 30 °C in phosphorylation buffer that contained one of several nucleotides plus one of the following preparations of recombinant PA-PLA1α that were bound to FLAGM2 beads: 1) a preparation that had been dephosphorylated by λ phosphatase; 2) a similar preparation that had first been dephosphorylated by λ phosphatase and then phosphorylated for 30 min at 30 °C with CK2α and subsequently treated with 350 mM KCl; or 3) a similar preparation of successively dephosphorylated and phosphorylated PA-PLA1α that had been treated with PP2A before being treated with 350 mM KCl. After the incubations, the beads were washed with buffer, and the amounts of CK2α that had bound to PA-PLA1α were determined (see “Experimental Procedures”). In A, the nucleotides used were MgATP, ATP-γ-S, or AMP-PNP; in B, the nucleotides were MgATP, MgGTP, MgADP, MgCTP, or MgUTP. Similar results were obtained in two experiments.

**Fig. 7.** Size exclusion chromatography of recombinant PA-PLA1α, GST-tagged CK2α, and a complex of the two enzymes. PA-PLA1α, GST-tagged CK2α, and a complex of PA-PLA1α plus GST-tagged CK2α were chromatographed separately on a 10/30 column of Superdex G 200, and positions of the proteins emerging in the effluent were monitored by dot blotting using antibodies to the enzymes followed by quantitative densitometry (see “Experimental Procedures”). Molecular masses of the proteins shown were calculated on the basis of a standard curve generated from experiments with bovine thyroglobulin (670 kDa), bovine γ-globulin (158 kDa), and chicken ovalbumin (44 kDa), whose elution positions are indicated by arrows.
the C-terminal region of PA-PLA1α to precipitate the phospholipase (see "Experimental Procedures"). Analysis of the PA-PLA1 activity in the precipitates using mixed micelle assays demonstrated that each one contained the enzyme (data not shown). Moreover, analysis by SDS-PAGE, silver staining, and Western blotting using antibodies to the predicted coiled-coil-forming region or C-terminal region of PA-PLA1α showed that each precipitate contained material that had an apparent molecular mass of 115 kDa (data not shown). On the other hand, analysis by SDS-PAGE, silver staining, and Western blotting using the antibodies to CK2α, CK2α', or CK2β showed that only the precipitate from pool 1 contained CK2α, whereas neither of the precipitates contained CK2α', CK2β, or other proteins that were detectable by silver staining (Fig. 8C and data not shown).

To confirm that the concentrated material from pool 1 contained a complex of PA-PLA1α plus CK2α, we used the antibody to CK2α to precipitate the kinase from the concentrate and then analyzed the material in the precipitate by SDS-PAGE and Western blotting using the antibodies to the coiled-coil-forming region and the C-terminal region of PA-PLA1α. As anticipated, the results demonstrated that PA-PLA1α-like material that had an apparent molecular mass of 115 kDa was present in the immune precipitate (data not shown).

To measure the relative distribution of PA-PLA1-like material in the two peaks obtained by size exclusion chromatography, we used the antibody to the C-terminal region of PA-PLA1α to precipitate enzyme-containing material from each of the concentrated pools and then examined the precipitates by SDS-PAGE and quantitative densitometry using additional aliquots of the antibody. The results of two independent experiments revealed that pool 1 contained about 40% of the total recovered enzyme and that about one-fourth of this was present as a complex with CK2α (data not shown).

We used a similar approach to determine whether high-speed supernatant fractions from homogenates of the frontal cerebral cortex of macaque brains also contain a complex of PA-PLA1α plus CK2α. Two separate size exclusion chromatography experiments showed that PA-PLA1α-like material emerged as a single peak of >670 kDa (Fig. 8B). Moreover, in each case, material precipitated from a concentrate of the corresponding fractions (with the use of the antibody to the C-terminal region of PA-PLA1α) could be shown to contain both phospholipase activity, as determined by the mixed micelle assay, and a protein that had an apparent molecular mass of 115 kDa, as determined by SDS-PAGE and Western blotting using antibodies to the coiled-coil-forming region or C-terminal region of PA-PLA1α (data not shown). Interestingly, however, no CK2α, CK2α', or CK2β could be detected by Western blotting with the respective antibodies (data not shown). The combined results of these experiments support the conclusions that 1) the testes of macaques contain large complexes of PA-PLA1 including a complex that also contains CK2α, and 2) the macaque cerebral cortex also contains a large complex of PA-PLA1-like material but no detectable complex that also contains CK2α. Further work will be required to determine the basis and functional significance of this apparent difference in tissue distribution.

**DISCUSSION**

The results of this study provide new information not only about the ability of PA-PLA1α to be phosphorylated and dephosphorylated in vitro but also about the enzyme's ability to form complexes with CK2αβ and its subunits, about the mechanism and effects of complex formation, and about the distribution of PA-PLA1α-containing complexes in macaque tissues. Furthermore, they identify a number of questions that warrant attention. One of these questions relates to the region of the phospholipase that contains serines 716 and 730. The fact that CK2 can phosphorylate serine 716, ERK2 can phosphorylate serine 730, there is cross-antagonism between these phosphorylation reactions, and PPP2A can catalyze the selective hydrolysis of phosphate groups that are esterified to the two serines raises the possibility that the native counterparts of these enzymes might coordinately regulate the phosphorylation state of this region in vivo. This possibility is of interest although the phosphorylation of serine 730 by ERK2 had no effect on the activity of PA-PLA1α in vitro, as mentioned previously. An alternate possibility that has to be considered is that the phosphorylation state of the two serines might affect some other property of the enzyme, such as the ability to bind to another cell protein. There is precedent for this type of regulation, because the CK2-dependent phosphorylation of serines 26 and 73 in caldesmon has been shown to reduce the ability of this substrate to bind myosin (15), the ERK2-dependent phosphorylation of serine 702 has been reported to reduce the ability of caldesmon to bind actin-tropomyosin (16), and the ERK2-dependent phosphorylation of serine 64 in another protein, PHAS-I, has been shown to reduce the ability of this substrate to bind to initiation factor 4E (17, 18).

Another question that warrants attention relates to the acidic, 28-amino-acid region of the phospholipase that contains serines 93 and 105. Our results show that CK2 phosphorylates both of these serines (Fig. 1, A and B) and raise the possibility that the phosphorylation reactions might influence the formation of a stable complex between the phospholipase and CK2α (Fig. 5, A and B; Table IV). But whether the phosphate groups that become esterified to serines 93 and 105 interact directly with the phospholipase remains to be determined. One way to explore this possibility might be to do incubation experiments with a synthetic, 28-amino-acid peptide that corresponds to the region of the phospholipase that contains the two serines. If this peptide turns out to be a good substrate for CK2α, the possibility that it might form a stable complex with the kinase could be tested by direct experimentation.

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2 Note that analysis by SDS-PAGE of PA-PLA1α that had been purified from bovine testes showed that the enzyme had an apparent molecular mass of ~110 kDa (4).

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**TABLE V**

Effects of phosphorylation by CK2α or ERK2 on the activity of PA-PLA1α

| Samples          | Mixed micelle assay | Vesicle assay |
|------------------|---------------------|---------------|
|                  | Enzyme activity ± S.E. | Activity | Enzyme activity ± S.E. | Activity |
|                  | pmol/min/μg protein | %       | pmol/min/μg protein | %       |
| PA-PLA1α + buffer| 315 ± 6.3           | 100      | 303 ± 8.7           | 100      |
| PA-PLA1α + CK2α  | 160 ± 1.6           | 51       | 168 ± 8.4           | 56       |
| PA-PLA1α + ERK2  | 308 ± 3.4           | 98       | 275 ± 14.7          | 92       |
Why MgATP or MgGTP is required for the formation of stable complexes between CK2α and prephosphorylated forms of PA-PLA1 also remains to be determined. One possibility is that MgATP or MgGTP might bind to the trinucleotide substrate-binding site on CK2α and have an allosteric effect on the kinase that promotes the exposure of one or more regions of the kinase that are required for the formation of a stable complex with the phosphorylated phospholipase. If a phosphorylated form of the above mentioned synthetic peptide forms a MgATP/MgGTP-dependent complex with the kinase, it might be possible to examine this potential mechanism directly with the use of NMR. Experimentation of this type would appear to be justified because similar ATP/GTP-dependent mechanisms might contribute to the formation of stable complexes between CK2α and its other protein substrates.

Our observation that the complex containing CK2α and PA-PLA1α behaves like a heterooctamer when it is analyzed by size-exclusion chromatography (Fig. 7) raises other questions. While PA-PLAα was being purified from bovine testis, size exclusion experiments provided evidence that it was a homotetramer (3). On the other hand, size exclusion experiments done in the present investigation showed that the dephosphorylated preparation of recombinant PA-PLA1α that we used was a homodimer (Fig. 7). The phospholipase contains a 28-amino acid, putative coiled-coil-forming region (4) that may influence the enzyme's ability to form homooligomers because model peptides that correspond to this region form amphipathic helices that interact hydrophobically (data not shown). But it is not clear why the enzyme forms homotetramers in some instances and homodimers in others or why the MgATP/MgGTP-dependent formation of a complex between CK2α and the phospholipase results in the apparent formation of a heterooctamer. The latter question deserves to be studied further because the presumptive heterooctamer showed reduced phospholipase activity (Table V), and the possibility has to be considered that the enzyme's ability to form large complexes with CK2α might...
be of regulatory importance in vivo (see below).

The large complexes of PA-PLA1 that contain no CK2α (Fig. 8, A and B) also warrant further study, though it is not yet clear that they have reduced activity. Some members of the phospholipase A2 superfamily also form soluble complexes (19–21). Furthermore, it has been proposed that clusters of phospholipase A2 in the cytoplasm of fibroblasts may form a localized, inactive pool from which active monomers can be recruited (21).

Whether the complex of CK2α and PA-PLA1 that we detected in homogenates of the macaque testis plays a special role in this organ is unclear. All that is known at present is that the mRNA for PA-PLA1 is expressed selectively in the spermatids in this organ is unclear. All that is known at present is that the mRNA for PA-PLA1 is expressed selectively in the spermatids of macaque testes and that the enzyme is present in bovine sperm. Therefore, it is reasonable to suppose that the complex between PA-PLA1 and CK2 may play a role in the differentiation of spermatids or in the function of sperm.

The differentiation of spermatids (spermiogenesis) is a fascinating process that involves the regulated translation of mRNA (22), the formation and storage of proteins for later use in assembly of the sperm tail (23), and impressive processes of polarization that are associated with major changes in the character and distribution of cytoplasmic organelles (24). These processes occur over a period of about 2 weeks, while the spermatids are bound to Sertoli cells. Therefore, it might be a challenging task to determine whether or how a complex of CK2α and PA-PLA1 might contribute to the specific molecular events that are involved. On the other hand, a study of the role and regulation of PA-PLA1 in sperm might be feasible. The biochemistry and cell biology of sperm have been studied extensively by others, evidence has been obtained that they contain CK2 and ERK2, and protein phosphorylation reactions have been shown to occur during sperm capacitation and the acrosome reaction (25, 26).

Acknowledgments—We thank Lynda Munar for help with the SF9 cell culture; Tyler Johnson for help with size exclusion chromatography; Marjorie Domenowske for the figures; Dr. Dongxia Li (Fibrogen, San Francisco, CA) for the CK2 constructs and antibodies; and Dr. Ken Applegate for advice on statistical analysis, modeling studies, and preparation of the manuscript.

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Effects of Protein Kinase CK2, Extracellular Signal-regulated Kinase 2, and Protein Phosphatase 2A on a Phosphatidic Acid-prefering Phospholipase A1
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J. Biol. Chem. 2001, 276:27698-27708.
doi: 10.1074/jbc.M101983200 originally published online April 27, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101983200

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