Phosphoinositide 3-Kinase in Rat Liver Nuclei†

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ABSTRACT: Biochemical and immunochemical data from the present investigation reveal the existence of a p85/p110 phosphoinositide 3-kinase (PI 3-kinase) in rat liver nuclei. ^{32}P-Labeling of membrane phosphoinositides by incubating intact nuclei with [γ-^{32}P]ATP results in the formation of [^{32}P]phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P_3], accompanied by small quantities of [^{32}P]phosphatidylinositol 3-phosphate [PtdIns(3)P]. Studies with subnuclear fractions indicate that the PI 3-kinase is not confined to nuclear membranes. The nuclear soluble fraction also contains PI 3-kinase and an array of inositide-metabolizing enzymes, including phospholipase C (PLC), phosphoinositide phosphatase, and diacylglycerol (DAG) kinase. As a result, exposure of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] to the nuclear extract in the presence of [γ-^{32}P]ATP generates a series of ^{32}P-labeled D-3 phosphoinositides and phosphatidic acid (PA) in an interdependent manner. On the basis of the immunological reactivity and kinetic behavior, the nuclear PI 3-kinase is analogous, if not identical, to PI 3-kinase α, and constitutes about 5% of the total PI 3-kinase in the cell. Moreover, we test the premise that nuclear PI 3-kinase may, in part, be regulated through the control of substrate availability by PtdIns(4,5)P_2-binding proteins. Effect of CapG, a nuclear actin-regulatory protein, on PI 3-kinase activity is examined in view of its unique Ca^{2+}-dependent PtdIns(4,5)P_2-binding capability. In vitro data show that the CapG-mediated inhibition of nuclear PI 3-kinase is prompted by PKC phosphorylation of CapG and elevated [Ca^{2+}]. This CapG-dependent regulation provides a plausible link between nuclear PLC and PI 3-kinase pathways for cross-communications. Taken together, these findings provide definite data concerning the presence of an autonomous PI 3-kinase cycle in rat liver nuclei. The nuclear location of PI 3-kinase may lead to a better understanding regarding its functional role in transducing signals from the plasma membrane to the nucleus in response to diverse physiological stimuli.

The existence of a discrete phosphoinositide signaling pathway in the nucleus has been the focus of many recent investigations (*1*, *2*). Substantial evidence indicates that nuclei contain many enzymes involved in the canonical phosphoinositide cycle, including kinases for the synthesis of PtdIns(4,5)P_2[1] (*3*), different isoforms of PLC (*4*−*6*), inositol phosphate-related phosphatases (*7*), and DAG kinase (*8*). It is noteworthy that the nuclear PLC pathway is susceptible to receptor activation at the plasma membrane without its cytoplasmic counterpart being affected. For example, stimulation of Swiss 3T3 cells with insulin-like growth factor led to the activation of the nuclear PLC β1, resulting in a decrease in the mass of PtdIns(4)P and PtdIns(4,5)P_2 with a concomitant increase in nuclear DAG (*9*). Further, this nuclear phosphoinositide breakdown was reported to be an S-phase-specific event in the cell cycle, implying the direct role of nuclear PLC signaling in cell proliferation (*7*). This premise is corroborated by the findings that the breakdown products of phosphoinositides, such as PtdIns(4)P (*10*), Ins(1,4)P_2 (*11*), and DAG (*9*), were involved, directly or indirectly, in the regulation of DNA synthesis. More recently, immunocytochemical evidence suggests the presence of another signaling enzyme PI 3-kinase in the nuclei of rat pheochromocytoma PC 12 cells (*12*) and human osteosarcoma Saos-2 cells (*13*). PI 3-kinase phosphorylates the D-3 position of PtdIns(4,5)P_2, PtdIns(4)P, and phosphatidylinositol (PtdIns) to generate the putative second messengers, PtdIns(3,4,5)P_3, phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P_2], and PtdIns(3)P, respectively, and has been linked to diverse cellular responses triggered by cell surface receptors (*14*−*17*). To date, at least three PI 3-kinase isozymes have been characterized and

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[1] Abbreviations: PI 3-kinase, phosphoinositide 3-kinase; PLC, phospholipase C; PKC, protein kinase C; SH 2 domains, Src homology 2 domains; PtdIns, phosphatidylinositol; PtdIns(3)P, phosphatidylinositol 3-monophosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(3,4,5)P_3, phosphatidylinositol 3,4,5-trisphosphate; PA, phosphatidic acid; PS, phosphatidylserine; DAG, diacylglycerol; Ins(1,4,5)P_3, D-*myo*-inositol 1,4,5-trisphosphate; Ins(1,4)P_2, D-*myo*-inositol 1,4-bisphosphate; GroPIns(3,4,5)P_3, glycerolphosphorylinositol 3,4,5-trisphosphate; GroPIns(3,4)P_2, glycerolphosphorylinositol 3,4-bisphosphate; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; tlc, thin-layer chromatography.

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cloned. PI 3-kinase isotypes α and β are heterodimers consisting of p85 regulatory subunits and p110 catalytic subunits. The p85/p110 PI 3-kinase is regulated by membrane-bound receptor tyrosine kinases through specific recognition of phosphotyrosine residues on the receptors by the SH2 domain of p85 (*15*). The third isotype, p110γ, was recently identified and reported to be activated by heterodimeric G protein coupled receptors (*18*).

Despite recent advances in exploring the physiological relevance of PI 3-kinase lipid products, information concerning the formation of these D-3 phosphoinositides in the nucleus is totally lacking in the literature. Earlier studies on nuclear phosphoinositide metabolism have failed to detect any of these novel inositol lipids (*3*, *19*, *20*). Thus, as part of our effort to understand the functional role of PI 3-kinase, we examined in detail the enzyme as well as its lipid products in rat liver nuclei. Our study indicates that rat liver nuclei contained an active D-3 phosphoinositide cycle that consists of a p85/p110 PI 3-kinase and related phosphatases. This metabolic pathway bears resemblance to its cytoplasmic counterpart, and in conjunction with the PLC pathway, generates a host of signaling molecules that might take part in the regulation of nuclear functions in a synergistic manner. Moreover, the present study suggests that CapG (*21*), a unique PtdIns(4,5)P_2-binding protein, may take part in the regulation of nuclear PI 3-kinase through substrate sequestration.

EXPERIMENTAL PROCEDURES

Materials. [1-^{3}H]PtdIns(3,4,5)P_3, [1-^{3}H]PtdIns(3,4)P_2, and [1-^{3}H]PtdIns(3)P were prepared according to a modification of the synthetic procedures previously described for the respective D-3 phosphoinositides (*22*). Other phospholipids including PtdIns(4,5)P_2, PtdIns(4)P, phosphatidylinositol (PtdIns), phosphatidylserine, and 1,2-dioctanoyl-*sn*-glycerol were purchased from either Sigma or Calbiochem. PKC-α was a product of PanVera Co. Rat liver PI 3-kinase was a kind gift of Professor Lewis C. Cantley. Monoclonal antibody against the p85 subunit of PI 3-kinase was obtained from Transduction Laboratory. Anti-β-tubulin monoclonal antibody was from Calbiochem. [^{3}H]PtdIns(4,5)P_2 and [γ-^{32}P]ATP were purchased from NEN Life Science Products. Protein A-Sepharose was obtained from Pharmacia.

Preparation of Rat Liver Nuclei. The preparation of rat liver nuclei was carried out by a modification of the method of Masmoudi et al. (*23*). All operations were performed at 4 °C. Rat livers were collected on ice, perfused with ice-cold 50 mM Tris/HCl, pH 7.5, containing 25 mM KCl and 5 mM $MgCl_2$, and cut into small pieces in ice-cold Buffer A, consisting of 1.3 M sucrose, 1 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8. After rinsing, the small pieces were homogenized in 8 vol of the same buffer in a Dounce homogenizer with six strokes up and down. The homogenate was filtered through four layers of cheesecloth and centrifuged at 1000*g* for 20 min. The resulting pellet was suspended in a minimum volume of ice-cold buffer A and homogenized again in a Dounce homogenizer with five strokes. The resulting suspension was mixed with 3.5 vol of ice-cold buffer B, consisting of 2.4 M sucrose, 1 mM $MgCl_2$, and 10 mM potassium phosphate, pH 6.8, and centrifuged at 100000*g* for 1 h. The clear, white nuclear pellet was suspended in an appropriate volume of ice-cold buffer C, containing 0.25 M sucrose, 1 mM $MgCl_2$, and 20 mM Tris/HCl, pH 7.5, and centrifuged at 1000*g* for 10 min. The washing was repeated twice to have the clear, white final nuclear pellet. The pellet was suspended in ice-cold buffer C for immediate use in the radiolabeling experiment. For the subfractionation study, the whole nuclei were suspended in a lysis buffer, containing 30 mM Hepes, pH 7.4, 0.25 M sucrose, 1 mM EDTA, 0.5 mM DTT, 200 nM 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), 10 μg/mL leupeptin, and 200 μM sodium vanadate. The suspension was sonicated for 1 min at 4 °C and centrifuged at 150000*g* for 90 min to yield the nuclear soluble and membrane fractions. Both fractions were immediately used for PI 3-kinase assay. It was found that freezing and thawing of the samples resulted in partial deactivation of PI 3-kinase while phosphatases were unaffected.

Nuclear Lipid Labeling. Rat liver nuclei (40 μg of protein), suspended in 100 μL of buffer C, were incubated with 1 mM ATP, 10 μCi of [γ-^{32}P]ATP, and 5 mM $MgCl_2$ for various intervals. The reactions were stopped by the addition of 5 μL of 1 M EDTA and 25 μL of 5 M HCl followed by 160 μL of chloroform/methanol (1:1; v/v). The phases were separated by centrifugation at 6000*g* for 5 min. The organic layer was dried by a stream of N_2, spotted onto 1% oxalic-acid-treated tlc plates, and then developed with *n*-propanol:2 M acetic acid (65:35) overnight (*24*). After drying, spots were located by autoradiography and compared with standards. The autoradiograms were scanned by a Photodyne image system and quantified using an NIH image program (version 1.59). In a separate experiment, the incubation was carried out under identical conditions, however, without the addition of [γ-^{32}P]ATP. At indicated intervals, aliquots were taken, and the nuclei were examined by light microscopy after trypan blue staining. No appreciable morphological change of the nuclei was noted throughout the 10 min incubation.

PI 3-Kinase Assay. PtdIns(4,5)P_2 (10 μg) and phosphatidylserine (PS) (40 μg) were suspended in 100 μL of 30 mM Hepes, pH 7.5, containing 1 mM EDTA and 1 mM EGTA, sonicated in a water bath-type sonicator for 5 min, and mixed vigorously with a vortex mixer before assays. Various PI 3-kinase preparations (10 μL) were incubated with 80 μL of 30 mM Hepes, pH 7.5, containing 125 μM ATP, 10 μCi [γ-^{32}P]ATP, and 6.25 mM $MgCl_2$. The reaction was initiated by adding 10 μL of the phospholipid solution, incubated at 37 °C for 10 min, and stopped by adding 5 μL of 1 M EDTA and 25 μL of 5 M HCl followed by 160 μL of chloroform:methanol (1:1; v/v). Procedures for the subsequent lipid extraction and tlc analysis were carried out as described above. In the substrate specificity experiment, PtdIns or PtdIns(4)P was used instead of PtdIns(4,5)P_2 as external substrate.

The authenticity of radiolabeled D-3 phosphoinositides was confirmed by HPLC analysis of the deacylated products in reference to glycerolphosphorylinositol phosphates derived from the deacylation of tritium-labeled standards including [^{3}H]PtdIns(3,4,5)P_3, [^{3}H]PtdIns(4,5)P_2, [^{3}H]PtdIns(3,4)P_2, and [^{3}H]PtdIns(3)P. Lipid deacylation was carried out by using the methylamine/methanol method as described (*25*). The deacylated products were analyzed by HPLC on a 4.6 × 200 mm Adsorbosphere Sax column (5 μ), equilibrated with H_2O

(solvent A) at a flow rate of 1 mL/min. A gradient with solvent B [0.5 M $NH_4H_2PO_4$, pH 3.9] was applied as follows: 0 to 10% solvent B in 5 min, 10 to 70% solvent B in 70 min, 70 to 100% solvent B in 10 min, and 100 to 0% for 10 min. Fractions were collected every 1 mL, and their radioactivity was measured by liquid scintillation.

Partial Purification of Nuclear PI 3-Kinase. The protocol was based upon Cantley's procedure for purifying cytosolic PI 3-kinase (*24*). (a) *Acid Precipitation*. The pH of the nuclear soluble fraction was adjusted to 5.7 with 2 M acetic acid. The precipitate was collected by centrifugation at 10000*g* and dissolved in buffer DEAE, consisting of 50 mM Tris/HCl, pH 7.5, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 1 mM AEBSF, and 1 mM DTT. (b) *DEAE-52 Chromatography*. The resulting solution from acid precipitation was applied to a column of DEAE-52 (2 × 10 cm) preequilibrated with buffer DEAE. Proteins were eluted with a liner gradient of 0 to 0.4 M NaCl in the same buffer. The active fractions were pooled and concentrated by ultrafiltration. (c) *Sephacryl S-200 Gel Filtration Chromatography*. The pooled active fractions were loaded onto a Sephacryl S-200 column (1 × 50 cm) preequilibrated with 100 mM Tris/HCl, pH 7.5, containing 1 mM DTT. The enzyme was washed with the equilibration buffer. Active fractions were pooled and concentrated by ultrafiltration.

Immunoblotting. Proteins from the cytosol and crude nuclear extracts containing both membrane and soluble fractions were separated by 12% SDS−PAGE on a Bio-Rad Minigel apparatus, and transferred to a nitrocellulose membrane using a Bio-Rad semi-dry transfer cell. The transblotted membrane was washed twice with TBS that consisted of 20 mM Tris/HCl, pH 8.0, and 137 mM NaCl. After blocking with TBS containing 5% low-fat milk for 20 min, the membrane was incubated with 0.1 μg/mL of mouse mAb against the p85 subunit of PI 3-kinase α (Transduction Laboratory; IgG1 isotype) in TBS-5% low fat milk at 4 °C for 12 h and then washed twice with TBS containing 0.05% Tween 20 (TBS/Tween 20). The membrane was then incubated with goat anti-mouse IgG-biotin conjugates for 1 h at room temperature and washed twice with TBS/Tween 20. The membrane was then incubated with avidin-horseradish peroxidase conjugates for 40 min, and washed twice with TBS/Tween 20. Visualization was carried out by exposing the membrane to 1.6 mM 3,3′-diaminobenzylidine in 50 mM Tris, pH 7.4, containing 0.03% H_2O_2.

Immunoadsorption. The aforementioned anti-p85 (5 μg) was added to 500 μL of the soluble nuclear fraction of which the protein concentration was adjusted to 1 μg/mL with PBS. The mixture was incubated with gentle rocking at 4 °C for 12 h, 100 μL of protein A-Sepharose was added, and the incubation was continued for another 2 h. The immunoprecipitates were collected by centrifugation and washed, in sequence, three times with PBS-1% NP-40, twice with 0.1 M Tris/HCl, pH 7.5, containing 0.5 M LiCl, and twice with 10 mM Tris/HCl, pH 7.5, containing 100 mM NaCl and 1 mM EDTA. The washed antibody conjugates were analyzed for PI 3-kinase activity as described above.

Immunoelectron Microscopy. The rat liver was perfused with 1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 1 h, cut into pieces, and incubated with the buffered glutaraldehyde for 30 min. After two washes in 0.1 M sodium cacodylate and postfixation in 0.1% osmium tetroxide, the tissue was dehydrated up to 100% ethanol-acetone (1:1) and embedded in Spurr's resin. Thin sections were preincubated with 10% goat serum in PBS for 1 h, incubated overnight with mouse anti-p85 monoclonal antibody (Transduction Laboratory; IgG1 isotype) in PBS (1:100 dilution), and treated with goat anti-mouse IgG conjugated with 10 nm of collidal gold in PBS (1:50 dilution) at room temperature for 1 h. The controls consisted of samples exposed to mouse IgG1 (Sigma) instead of anti-p85, followed by the same treatment.

Phosphorylation of CapG by PKC α. Recombinant CapG was expressed and purified as described (*26*). Twenty micromolar CapG, in a reaction volume of 250 μL, was incubated with 40 ng of PKC α at 30 °C in the presence of 30 mM Hepes, pH 7.5, 200 μM $CaCl_2$, 10 mM $MgCl_2$, 0.5 mM ATP, 25 μg of phosphatidylserine, 5 μg of dioctanoylglycerol, and 0.02% Triton X-100. In a parallel experiment, an identical incubation with $^1/_{10}$ of the aforementioned scale was carried out in the presence of 5 μCi [γ-^{32}P]ATP as a tracer to assess the extent of phosphorylation by two methods. First, aliquots were applied onto phosphocellulose membrane units (Pierce) and centrifuged. The membrane was washed with 75 mM H_3PO_4 twice, and the membrane-associated radioactivity was determined by liquid scintillation. Second, aliquots were subjected to SDS−PAGE followed by autoradiography.

After 4 h, the large-scale reaction was terminated by applying the mixture onto a Sephacryl S-200 column (1 × 25 cm), preequilibrated with 50 mM NH_4OAc, pH 7.0, containing 1 mM DTT, to separate phospho-CapG from lipid vesicles, lipid-anchored PKC α, and ATP. Fractions containing CapG were collected, lyophilized, and reconstituted with 40 mM Hepes, pH 7.4, containing 1 mM DTT, in a final concentration of 40 μM. Protein concentrations were determined by using a Comassie protein assay reagent (Bio-Rad) with BSA as a standard. In a control experiment, CapG was subjected to the same treatments described above except that PKC α was not added in the incubation. The resulting CapG was used as a control in the PI 3-kinase assay described below.

Effect of CapG and Phospho-CapG on PI 3-Kinase Activity. CapG or phospho-CapG was preincubated with PtdIns(4,5)P_2 for 30 min with or without Ca^{2+} before partially purified nuclear PI 3-kinase was added. The incubation mixture consisted of 30 mM Hepes, pH 7.5, various amounts of CapG or phospho-CapG, 5 μM PtdIns(4,5)P_2, PtdSer (4 μg), 0.2 mM EGTA, 0.2 mM EDTA, 5 mM $MgCl_2$, and 0.1 mM ATP in the presence or absence of 0.28 mM $CaCl_2$. Accordingly, free [Ca^{2+}] and [Mg^{2+}] were calculated to be 24.6 μM and 4.76 mM, respectively, in the EDTA, EGTA-containing buffer. The procedure for the PI 3-kinase assay was identical to that aforementioned.

RESULTS

Purity of Nuclear Preparations. Rat liver nuclei were prepared as described by Malviya and co-workers (*23*). In addition to the microscopic examination of the toluidine blue-stained nuclei, the purity of the nuclear preparation was verified by biochemical and immunochemical analyses. Lactate dehydrogenase and alkaline phosphodiesterase activities, recognized as markers for cytoplasm and plasma

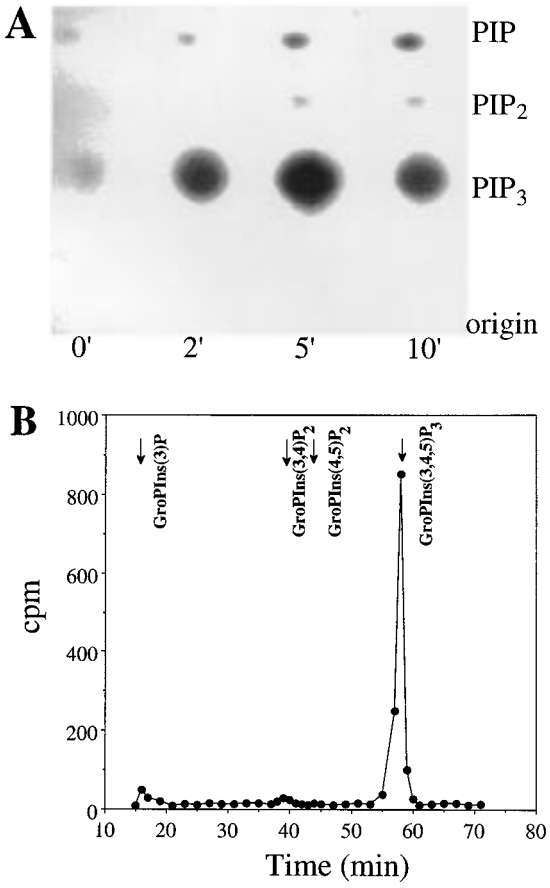

FIGURE 1: (A) Time course of ^{32}P-labeling of membrane phosphoinositides in intact rat liver nuclei, as represented by the autoradiogram of tlc-separated phospholipid extracts. The intact nuclei were exposed to [γ-^{32}P]ATP in a sucrose-containing Tris/HCl buffer for the indicated times (0, 2, 5, and 10 min). The subsequent lipid extraction and tlc analysis are described in Experimental Procedures. The autoradiogram is representative of three experiments. (B) HPLC profile of deacylated phospholipids extracted from the 5 min sample of ^{32}P-labeled nuclei. [^{32}P]-Phospholipids were deacylated by methanolysis and subjected to HPLC and compared with the elution of ^{3}H standards as described in Experimental Procedures. Tritium-labeled glycerolphosphatidylinositol 3-phosphate [GroPIns(3)P], GroPIns(3,4)P_2, GroPIns-(4,5)P_2, and GroPIns(3,4,5)P_3 were prepared from the respective phosphoinositides by the same procedure.

membrane (*27*), were found to be negligible in these preparations (<0.01% of that in the respective fractions). Moreover, Western blotting with anti-β-tubulin antibodies revealed the absence of β-tubulin, a cytoplasmic marker (*28*), from the nuclear preparation (data not shown).

Phosphorylation of Membrane Phosphoinositides in the Whole Nuclei. The purified nuclei were exposed to [γ-^{32}P]-ATP for various time intervals in a sucrose-containing buffer. Microscopic examinations of the nuclei after trypan blue staining indicated that the structural integrity was preserved over the period of incubation. ^{32}P-Labeled lipids derived from the phosphorylation of endogenous substrates were extracted and analyzed by tlc. Incorporation of radioactivity from [γ-^{32}P]ATP into membrane phosphoinositides was noted in a time-dependent manner (Figure 1A). The principal radiolabeled phospholipid was found to coincide with [^{32}P]PtdIns(3,4,5)P_3. The identity of the major product was further verified by subjecting the lipid to deacylation, followed by HPLC analysis of the resulting glycerolphosphorylinositol trisphosphate [GroPIns(3,4,5)P_3] (Figure 1B).

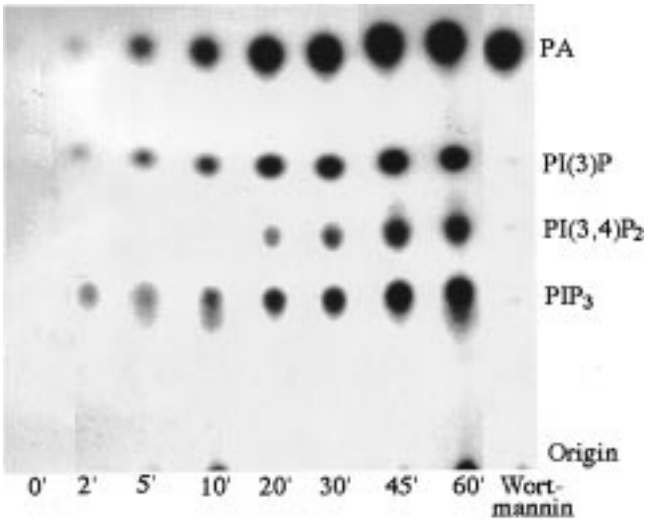

FIGURE 2: PtdIns(4,5)P_2 metabolism by the nuclear soluble fraction, as represented by the autoradiogram of tlc-separated phospholipid extracts. The nuclear soluble fraction was incubated with PtdIns-(4,5)P_2 vesicles in the presence of [γ-^{32}P]ATP for the indicated times (0, 2, 5, 10, 20, 30, 45, and 60 min). In a parallel experiment, a mixture of the same compositions was incubated with 100 nM wortmannin for 30 min. The phospholipids were extracted and analyzed by tlc as described in Experimental Procedures. The autoradiogram is representative of three experiments.

As shown, the production of [^{32}P]PtdIns(3,4,5)P_3, which peaked at approximately 5 min, was accompanied by a gradual, though small, accumulation of ^{32}P-labeled phosphatidylinositol monophosphate. In contrast, the formation of phosphatidylinositol bisphosphates including PtdIns(4,5)-P_2 and PtdIns(3,4)P_2 was negligible.

PI 3-Kinase and Phosphoinositide-Metabolizing Enzymes Localize to the Nucleus. To define the subnuclear locality of PI 3-kinase, the nuclei were sonicated and centrifuged at 150000*g* to yield nuclear soluble and membrane fractions. Examination of the enzyme activity with exogenous PtdIns-(4,5)P_2 as substrate indicated that the kinase resided in both soluble fraction and nuclear membranes, and that, besides PI 3-kinase, there existed several phosphoinositide-metabolizing enzymes in the nuclei. The profiles of phosphoinositide labeling in both fractions were analogous, which were represented in Figure 2.

As shown, short-term exposure of PtdIns(4,5)P_2 to the soluble fraction in the presence of [γ-^{32}P]ATP yielded ^{32}P-labeled PtdIns(3,4,5)P_3, PtdIns(3)P, and phosphatidic acid (PA). ^{32}P incorporation into these phospholipids increased over a 60 min time course. It is worthy to note that the emergence of PtdIns(3,4)P_2 lagged behind that of other D-3 phosphoinositides by about 20 min.

To examine the catalytic property of the nuclear PI 3-kinase, the soluble fraction was subject to partial purification by acid precipitation, DEAE-Sepharose chromatography, and S-200 gel filtration to remove competing phosphatases. These procedures resulted in an approximately 100-fold purification of the enzyme. The partially purified PI 3-kinase, devoid of phosphatase activities, catalyzed the conversion of PtdIns(4,5)P_2, PtdIns(4)P, and PtdIns to their respective D-3 phosphoinositides with similar potencies (Figure 3, lane B). This broad substrate specificity was consistent with the kinetic behavior of its cytosolic counterpart, as indicated in Figure 3 (lanes A) and that reported by Barnett et al. (*29*).

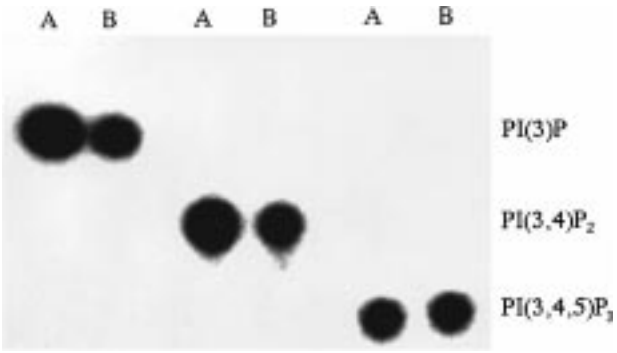

FIGURE 3: Substrate specificity of purified rat liver cytosolic PI 3-kinase α (lanes A) vs partially purified rat liver nuclear PI 3-kinase (lanes B). PtdIns, PtdIns(4), and PtdIns(4,5)P_2 were used as substrates to examine the PI 3-kinase activity as described in Experimental Procedures. The respective products were PtdIns(3)P, PtdIns(3,4)P_2, and PtdIns(3,4,5)P_3. The autoradiogram is representative of three experiments.

Immunochemical Examinations of Nuclear PI 3-Kinase. An equal amount (900 ng) of proteins from the cytosol and crude nuclear homogenates were separated by 12% SDS−PAGE, and the immunoblot analysis with anti-p85α monoclonal antibody revealed a 85 kDa protein in both the nuclear and cytosolic fractions (Figure 4A; lanes 1 and 2, respectively). This finding was verified by the immunoprecipitation from the soluble nuclear fraction using anti-p85α coupled to protein A beads. The immunoadsorbed proteins displayed PI 3-kinase activity that could be inhibited by wortmannin (Figure 4B). These data showed that the nuclear PI 3-kinase consisted of a p85 regulatory subunit. The catalytic subunit was presumably similar to p110.

In addition, the relative distribution of PI 3-kinase in the nucleus versus the cytosol was estimated from the relative intensity in the immunoblot (Figure 4A) and the respective total protein contents. Accordingly, the amount of nuclear p85 PI 3-kinase was calculated to be approximately 5.1% of that of its cytosolic counterpart, which was in agreement with that of 5.5% estimated from the PI 3-kinase activity in each fraction.

The nuclear localization of PI 3-kinase was further confirmed by electron microscope immunocytochemistry. The thin section of rat liver was immunostained with anti-p85 monoclonal antibody (IgG1 isotype) or treated with mouse IgG1 as control, followed by anti-mouse IgG-gold conjugates. As shown, the immunogold labeling clearly indicated the presence of PI 3-kinase in both the nuclear membrane and nucleoplasm (Figure 5A), which was absent in the control (Figure 5B). In comparison, the labeling appeared to be more densely distributed in the cytosol than in the nucleus.

A Plausible Mechanism for Nuclear PI 3-Kinase Regulation. A relevant issue that warranted discussion was the mode of PI 3-kinase regulation inside the nucleus. In this respect, we turned our attention to a unique actin-regulating protein, CapG, in the nucleus. CapG bound PtdIns(4,5)P_2 in a Ca^{2+}-dependent manner (*21*), with K_d values of 8.4 and 32 μM with and without Ca^{2+}, respectively (*30*). Thus, CapG might inhibit PtdIns(4,5)P_2-utilizing enzymes in a manner similar to that of profilin- or gelsolin-mediated PLC-γ1 inhibition, i.e., substrate sequestration. It is also noteworthy that nuclear CapG is preferentially phosphorylated

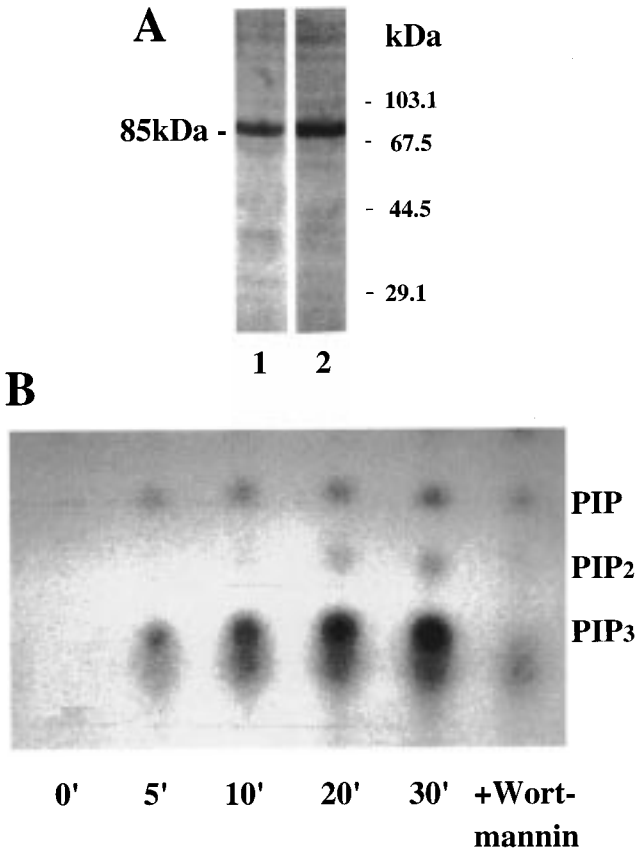

FIGURE 4: Immunochemical detection of PI 3-kinase in rat liver nuclei using mouse anti-p85 mAb. (A) Western blotting analysis of nuclear membrane and soluble fractions (lane 1) as well as cytosolic proteins (lane 2). (B) Time course of the conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3 by immunoadsorbed PI 3-kinase from the nuclear soluble fraction. The time intervals were 0, 5, 10, 20, and 30 min. In a parallel experiment, a mixture of the same composition was incubated with 100 nM wortmannin for 30 min. The phospholipids were extracted and analyzed by tlc as described in Experimental Procedures. The autoradiogram is representative of two experiments.

vis-à-vis its cytosolic counterpart (*31*). The contents of phospho-CapG in the soluble cytoplasm and nucleus were reported to be 7 and 46%, respectively, of total CapG in each fraction.

In light of these considerations, activity of the partially purified nuclear PI 3-kinase was analyzed in the presence of CapG alone, CapG with Ca^{2+}, or phospho-CapG with Ca^{2+}. The latter two simulated the physiological conditions when nuclei were activated.

First, phospho-CapG was prepared by incubating the parent protein with PKC α, and the extent of phosphorylation was confirmed by [γ-^{32}P]ATP tracing and SDS−PAGE/autoradiography (Figure 6). On the basis of the amount of ^{32}P incorporation into phospho-CapG, it was estimated that on average, there were four molecules of phosphates for each protein molecule.

PKC α was selected for CapG phosphorylation in view of a recent report that, among various PKC isozymes tested (α, β1, ϵ, and ζ), only the α isoform underwent intranuclear localization in Swiss 3T3 cells (*32*). In the present study, the PKC phosphorylation of CapG appeared to be independent of PtdIns(4,5)P_2. In contrast, a recent report by De Corte et al. (*33*) indicated that the phosphorylation of CapG and other actin-regulating proteins by tyrosine kinase $PP60^{c-src}$

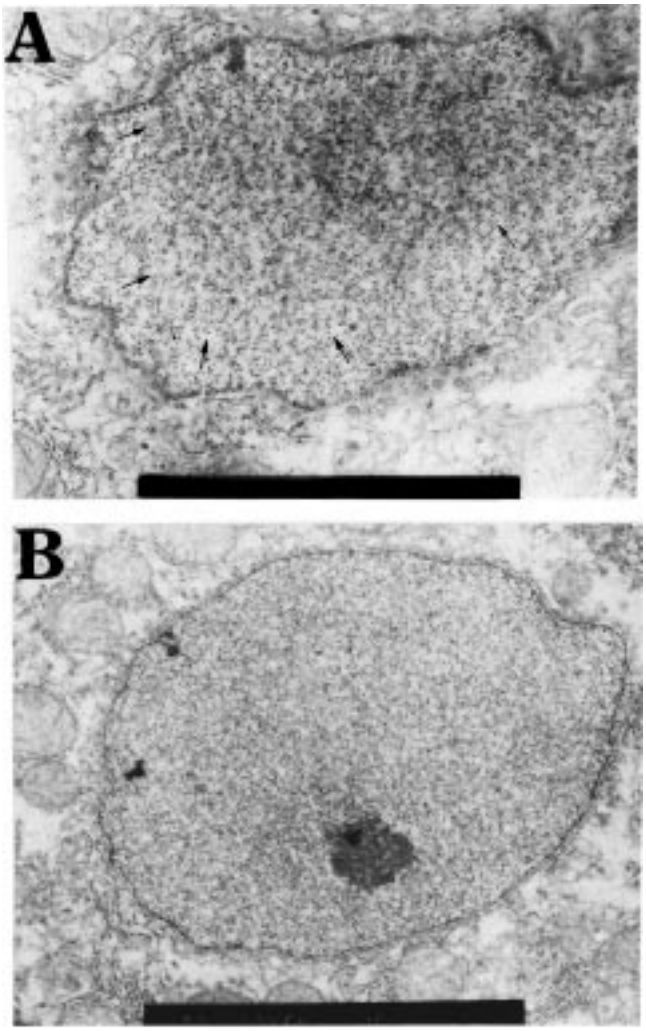

FIGURE 5: Electron micrographs (24000×) of rat liver nucleus (A) immunostained with anti-p85 monoclonal antibody or (B) treated with mouse IgG1control, followed by anti-mouse IgG-gold conjugates. Representative immunogold labelings are indicated by arrows in Panel A. The black bars represent the width of 5.87 μm.

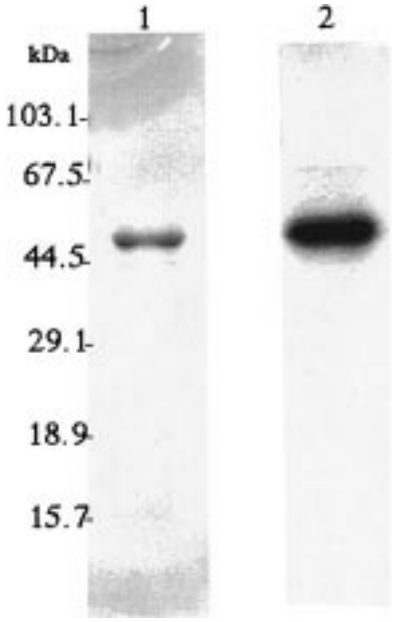

FIGURE 6: Phosphorylation of CapG by PKC α. Lane 1, Coomassie blue staining of CapG; lane 2, autoradiogram of [^{32}P]phospho-CapG. Phosphorylation did not cause any change in the electrophoretic mobility on SDS−PAGE.

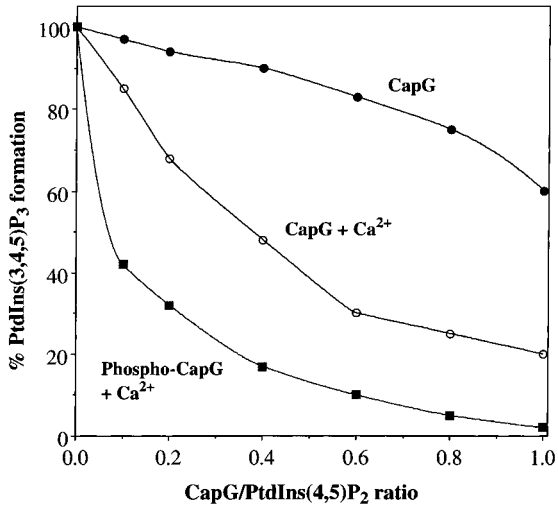

FIGURE 7: Dose-dependent inhibition of PI 3-kinase by CapG alone (full circle), CapG plus 24.7 μM Ca^{2+} (open circle), and phospho-CapG plus 24.7 μM Ca^{2+} (close square). PI 3-kinase activities under different conditions were analyzed as described in Experimental Procedures. Each data point represents the mean of three determinations.

was PtdIns(4,5)P_2 dependent.

Figure 7 shows the differential inhibition of nuclear PI 3-kinase among the three different conditions. It is noteworthy that phospho-CapG in combination with 100 μM Ca^{2+} significantly diminished the activity of PI 3-kinase, while CapG alone affected the activity to the least extent. In the presence of Ca^{2+}, nuclear PI 3-kinase was significantly inhibited even at low phospho-CapG/PtdIns(4,5)P_2 ratios. The extents of inhibition were 60, 68, and 83% at the ratios of 0.1, 0.2, and 0.4, respectively, while the corresponding values were considerably lower for CapG with Ca^{2+} or CapG alone. This dose-dependent inhibition might be attributed to the binding of CapG and phospho-CapG to PtdIns(4,5)P_2, which blocked the access of PI 3-kinase to its substrate. Presumably, phospho-CapG exerted higher degree of inhibition owing to its higher affinity with PtdIns(4,5)P_2 compared to that of CapG. This possibility is currently under investigation.

DISCUSSION

This study represents the first evidence that, besides the canonical phosphoinositide turnover pathway, rat liver nuclei contain an active D-3 phosphoinositide cycle that generates PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, and PtdIns(3)P in an interdependent manner. On the basis of the immunological reactivity and catalytic behavior, the nuclear PI 3-kinase was analogous, if not identical, to its cytoplasmic counterpart, PI 3-kinase α. It is noteworthy that the nuclear PI 3-kinase is present not only in the membrane but also in intranuclear locations along with phosphoinositide phosphatases. This finding conforms to the report that the main components of the canonical pathways, including PtdIns(4,5)P_2, PLC β1, PtdIns 4- and 5-kinases, and DAG kinases, reside at intranuclear sites, especially nuclear matrices (*34*, *35*).

These two phosphoinositide pathways, in conjunction with phosphatases and DAG kinase, transform PtdIns(4,5)P_2 to a host of metabolites. As shown in Figure 2, a brief exposure

of PtdIns(4,5)P_2 to nuclear extracts in the presence of [γ-^{32}P]-ATP led to the concurrent production of ^{32}P-labeled PtdIns-(3,4,5)P_3, PtdIns(3)P, and PA. Conceivably, besides PI 3-kinase catalysis, the exogenous PtdIns(4,5)P_2 was rapidly transformed by 5- and 4-phosphatases, in tandem, to PtdIns that was then converted to PtdIns(3)P by PI 3-kinase. Concomitantly, PLC-mediated hydrolysis of PtdIns(4,5)P_2 afforded DAG that was phosphorylated to PA by nuclear DAG kinase (*8*). The lagged formation of PtdIns(3,4)P_2 was likely to arise from PtdIns(3,4,5)P_3 hydrolysis by a specific 5-phosphatase. In addition, the synthesis of D-3 phosphoinositides, but not PA, was inhibited by wortmannin, confirming the susceptibility of nuclear PI 3-kinase to wortmannin inhibition.

Previously, an immunocytochemical study by Neri et al. (*12*) indicated that PI 3-kinase was translocated into the nucleus of PC 12 cells after agonist stimulation, while no nuclear association of the enzyme could be detected prior to the activation. This is in contrast to the present study that rat liver nuclei contained PI 3-kinase even in the absence of external stimuli. Moreover, earlier studies on nuclear phosphoinositide metabolism indicated that PtdIns(3,4,5)P_3 and other D-3 phosphoinositides were not produced in the nuclei of Friend cells (*3*). Such discrepancy may be attributed to difference in cell types, which is currently under investigation.

The nuclear location of PI 3-kinase and phosphoinositide-metabolizing phosphatases expands our current understanding concerning nuclear phosphoinositide metabolism. In the literature, some of the metabolites from the PLC pathway such as Ins(1,4,5)P_3, DAG, Ins(1,4)P_2, and Ins(4)P have been correlated with the modulation of nuclear Ca^{2+} or DNA synthesis (*7*, *9*−*11*). In light of the diversity of enzymes involved in nuclear D-3 inositol lipid metabolism, the PI 3-kinase pathway is also thought to be important in nuclear functions. For example, nuclear PI 3-kinase and its lipid products may provide a putative link for the communication between cytosolic and nuclear signaling pathways. Evidence shows that many SH2 or PH domain-containing signaling proteins display high affinity with PtdIns(3,4,5)P_3 or PtdIns-(3,4)P_2. These include the p85 subunit of PI 3-kinase (*36*), PLC-γ1 (*37*), *Akt* (*38*−*40*), and the nonreceptor Bruton's tyrosine kinase (*Btk*) (*41*, *42*). This specific peptide-phospholipid recognition constitutes a basis for the recruitment and/or activation of target proteins at the plasma membrane (*43*, *44*). Accordingly, it is plausible that the D-3 phosphoinositides on the nucleus may facilitate nuclear localization or association by recruiting proteins with PtdIns-(3,4,5)P_3- or PtdIns(3,4)P_2-binding motifs to nuclear membranes.

The existence of two metabolic pathways for phosphoinositides in the nucleus raises questions about how they are linked together to facilitate cross-talk. As more PtdIns(4,5)-P_2-binding proteins with important signaling functions are identified, the possibility of cross-talk between divergent PtdIns(4,5)P_2-binding proteins through regulation of substrate availability is particularly relevant. This has been well demonstrated in the cytoplasm. Ca^{2+}-regulated actin binding proteins in the gelsolin family, including gelsolin and CapG, bind PtdIns(4,5)P_2 (*30*, *45*) and inhibit PLC in vitro (*46*, *47*) and in vivo (*48*, *49*). Profilin, another cytoplasmic actin-binding protein, has also been shown to downregulate PLC-γ1 via PtdIns(4,5)P_2 sequestration (*50*). This inhibitory effect could be overcome by the phosphorylation of PLC-γ1 by tyrosine kinases (*51*). Taken together, these results suggest that PtdIns(4,5)P_2-binding skeletal proteins can modulate phosphoinositide signaling at the level of substrate availability as well as in cytoskeletal remodeling.

We tested the possibility that this mechanism could also operate in the nucleus. CapG is one of the few PtdIns(4,5)-P_2-binding actin-regulatory proteins identified thus far. Nuclear CapG is preferentially phosphorylated compared with cytosolic CapG (*31*). The functional consequence of the phosphorylation, however, has not been studied. Here, we compared the potency of native and phosphorylated CapG in inhibiting nuclear PI 3-kinase. Evidence indicates that phospho-CapG in concert with Ca^{2+} was more potent than the native protein in PI 3-kinase inhibition. Presumably, the binding to PtdIns(4,5)P_2 blocked the access of PI 3-kinase to its substrate. Although information concerning the concentrations of nuclear PtdIns(4,5)P_2 and phospho-CapG is lacking in the literature, our data show that even at low protein:lipid ratios, phospho-CapG in concert with Ca^{2+} significantly inhibited PI 3-kinase by substrate sequestration.

In summary, we have demonstrated that rat liver nuclei contain an autonomous PI 3-kinase pathway. The fact that the nuclear PI 3-kinase exists in both membranes and intranuclear locations suggests that it plays a diverse role in the regulation of nuclear functions. Plausible utilities of nuclear PI 3-kinase include the facilitation of nuclear localization and the anchoring of nuclear proteins in nuclear matrices. Also, it remains to be explored how cellular activators affect the partitioning of PI 3-kinase between the cytoplasm and the nucleus. Investigations are currently under way to address these questions.

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