A Differential Location of Phosphoinositide Kinases, Diacylglycerol Kinase, and Phospholipase C in the Nuclear Matrix*

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Several enzymes involved in the phosphoinositide metabolism have been shown to be present in nuclei of rat liver and Friend cells. In this paper we demonstrate that nuclear matrices of mouse NIH 3T3-fibroblasts and rat liver cells, isolated by nuclease treatment and high salt extraction, contain phosphatidylinositol 4-kinase (PtdIns 4-kinase), phosphatidylinositol 4-phosphate 5-kinase (PtdIns(4)P 5-kinase), diacylglycerol kinase, and phospholipase C. By a selective extraction the nucleus can be dissected in the peripheral matrix (lamina-pore complex) and the internal matrix as shown by using marker antibodies. Surprisingly, PtdIns 4-kinase was found exclusively in the peripheral nuclear matrix, whereas PtdIns(4)P 5-kinase was found to be associated to internal matrix structures. Diacylglycerol kinase and phospholipase C activities were also preferentially detected in the internal matrix. These data demonstrate a differential localization of the phosphoinositide kinases in the nucleus and suggest that the phosphoinositide metabolism may play a specific role in the nucleus.

Despite the fact that phosphoinositides are a minor fraction of membrane phospholipids they play a crucial role in the production of second messengers such as inositol triphosphate and diacylglycerol (1). The signal transduction cascade, known as the phosphoinositide pathway, leads to the activation of protein kinase C and calcium release from intracellular stores (2, 3). In addition to their role in signal transduction the phosphoinositides have been demonstrated to be able to modify the organisation of cytoskeletal proteins (4). It has also been suggested recently that several compounds derived from the phosphoinositide cycle are able to influence intranuclear processes. PtdIns(4,5)P_2 and inositol 1,4,5-trisphosphate have been demonstrated to activate in vitro the low specific activity form of DNA polymerase α (5), while an inositol 1,4,5-trisphosphate-sensitive Ca^{2+} pool has been found in rat liver nuclei (6, 7). Finally, the presence of inositol kinase C in the nucleus (8, 9) suggests a role for the phosphoinositide metabolism in the activation of the nuclear protein kinase C.

Synthesis of polyphosphoinositides has already been shown to occur in nuclei from Friend cells, suggesting that the responsible enzymes are located in the nucleus (10). Studying the same model, Cocco et al. (11) provide evidence for changes in \[^{32}P\]-labeling from \[^{32}P\]-ATP of PtdInsP_5 in nuclei, dependent on the state of differentiation of the cells. In addition, growth factors seem to be able to modify the phosphoinositide metabolism in nuclei from NIH 3T3 cells (12), suggesting a subtle regulation during cell activation. Thus, the enzymes from the phosphoinositide metabolism may be regulated by specific stimuli in the nucleus as well as in the plasma membrane (13, 14).

In this study we have investigated in more detail the association of enzymes involved in the phosphoinositide metabolism to the nuclear matrix. The nucleus is a highly organized organelle in which specific functions are located in discrete nuclear domains (15). After treatment of nuclei with detergent, nuclease, and high salt, a protein network is obtained which is designated as the nuclear matrix (16). The nuclear matrix can be divided further into two compartments, the internal matrix which contains nuclear elements (16) and the peripheral matrix or lamina-pore complex (17, 18). Enzymes involved in several nuclear functions, such as replication and transcription, have been found to be associated to these matrices (19-21).

Nuclei from both tissue and cultured cells can be isolated at high purity. Several marker enzymes have been used to prove this purity, and enzyme activity of plasma membrane markers as 5'-nucleotidase and Na^+\textsubscript{}\textsuperscript{}K^+\textsubscript{}ATPase has been found to be less than 1% in the nuclear fraction (11, 22). In contrast with these enzyme markers, isolated nuclei are frequently contaminated with cytoskeletal elements, especially with intermediate filaments (23, 24). Association of intermediate filaments has been visualized with different electron microscopical methods (25, 26) and it has been shown by Geergrats and Blobel (27) that vimentin is able to bind directly to lamin B, a component of the lamina-pore complex.

In this paper we describe our investigations on the location of PI kinases, DAG kinase, and PLC in the nuclear matrix from both NIH 3T3 fibroblasts and rat liver cells. Nuclei were isolated following different isolation protocols and both nuclear preparations were found to be contaminated with different cytoskeletal elements. Comparing the results obtained with nuclei from 3T3 cells and rat liver cells allows us to exclude a possible influence of both the isolation procedure.

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The abbreviations used are: PtdInsP, phosphatidylinositol monophosphate; PtdIns, phosphatidylinositol; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(4)P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; DAG, diacylglycerol; PBS, phosphate-buffered saline; Pipes, piperazine-N,N'-(2-ethanesulfonic acid); HPLC, high performance liquid chromatography; PI, phosphatidylinositol; lm, internal nuclear matrix; PeM, peripheral nuclear matrix; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PLC, phosphatidylinositol-specific phospholipase C.
and cytoskeletal contamination on our measurements. Here we demonstrate that isolated nuclear matrices from both NIH 3T3 fibroblasts and rat liver cells contain PtdIns(4) kinase, PtdIns(4,5) P 5-kinase, DAG kinase, and PLC activities. Dissection of nuclear matrices showed that the peripheral matrix (lamina-pore complex) contains PtdIns(4) kinase activity and traces of DAG kinase while the internal matrix contains PtdIns(4,5) P 5-kinase and DAG kinase. PLC activity was found to be enriched in the internal matrix. Our results confirm that several enzymes from the phosphoinositide metabolism are present in the nucleus and show that they are tightly associated to the nuclear matrix. Furthermore, we provide biochemical evidence for a differential location of phosphoinositide kinases, DAG kinase, and PLC in the nucleus, suggesting a role of the nuclear matrix in the compartmentalization of these enzymes in the nucleus.

**EXPERIMENTAL PROCEDURES**

**Materials**—Antibodies directed against lamin A/C (41CC4), vimentin (RMV), and tubulin (RC04) were a generous gift from F. C. S. Ramaekers (University of Limburg, The Netherlands). The antibody directed against the internal matrix protein p160 (AM 88) was a gift of Dr. R. Van Driel (E. C. Slater Institute, University of Amsterdam, The Netherlands). Antibodies against actin were obtained from Amersham (Amersham International, United Kingdom (U.K.)). Rat tail collagen was purchased from Sigma (St. Louis, U. S. A.). All other chemicals used, including lipids, were obtained from Sigma.

**Cell Culture**—Mouse NIH 3T3 fibroblasts were grown in Dulbeccco's modified Eagle's medium supplemented with 7.5% fetal calf serum (Integro, Zaandam, The Netherlands) in a 7% CO2 humidified atmosphere at 37 °C. Cells were grown to near confluency in 150-cm2 culture flasks (Costar, Cambridge, MA).

**Isolation of Membrane-depleted Nuclei—**Membrane-depleted nuclei from NIH 3T3 fibroblasts were isolated essentially as described by Fey et al. (28). Briefly, cells were washed twice with phosphate-buffered saline (PBS) at 37 °C and suspended by incubation for 5 min in 2 mM EGTA in PBS at 37 °C. After centrifugation for 5 min at 1,000 × g, the cells were resuspended and incubated for 7 min at 4°C in CSK 100 buffer (100 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 1% Triton X-100, 0.5 mM CaCl2, 10 mM Pipes, 1.2 mM phenylmethyisulfonyl fluoride, pH 6.8) in a concentration of 107 cells/ml. Membrane-depleted nuclei were pelleted at 650 × g for 5 min at 4°C. Rat liver nuclei were isolated from 3-month-old male Wistar rats (250-300 g) according to Izzurralde et al. (29). The nuclei were incubated for 7 min in ice-cold CSK 100 buffer, and the membrane-depleted nuclei were pelleted at 650 × g for 5 min. Nuclei from both NIH 3T3 fibroblasts and rat liver cells were finally washed twice with CSK 50 buffer (50 mM NaCl, 300 mM sucrose, 3 mM MgCl2, 10 mM Pipes, 1.2 mM phenylmethyisulfonyl fluoride, pH 6.8) and stored at -20°C.

**Nuclear Matrix Isolation**—Nuclear matrices were isolated from membrane-depleted nuclei as described by Fey et al. (28) with some minor modifications. The membrane-depleted nuclei were resuspended and incubated for 20 min at 37 °C in CSK 50 buffer with 100 μg/ml DNase I and 50 μg/ml RNase A (Boehringer, Mannheim, Federal Republic of Germany (F. R. G.)) (30). Subsequently, the chromatin associated proteins were released by adding dropwise 2 M ammonium sulfate to a final concentration of 0.25 M. After 15 min of incubation on ice the nuclear matrices were pelleted at 2,000 × g for 10 min on a cushion containing 45% glyceral and 5 mM sucrose in PBS. The matrices were collected with a pipette and diluted with CSK 50 to a final concentration of approximately 2 mg/ml.

**Isolation of Internal and Peripheral Matrices**—For the isolation of the internal matrix, membrane-depleted nuclei were first stabilized by incubation for 1 h with 0.5 mM sodium tetrathionate in 40 mM buffer on ice as described (16, 17). Nuclei were washed two times with CSK 50 buffer and digested with nucleases (100 μg/ml DNase I and 50 μg/ml RNase A) for 20 min at 37°C. Ammonium sulfate was added dropwise to a final concentration of 0.25 M as described above. Nuclei and cytoskeleton were resuspended and incubated for 20 min at 37°C in CSK 50 buffer containing 0.25 M ammonium sulfate and 40 mM dithiothreitol. The solubilized internal matrix was cleared from peripherar matrix by pelleting the peripheral matrix at 10,000 × g for 10 min. For the isolation of the peripheral matrix (16, 17), nuclear matrices were incubated for 20 min at 37°C in CSK 50 buffer supplemented with 0.25 M ammonium sulfate and 40 mM dithiothreitol. The peripheral matrix was pelleted by centrifugation for 10 min at 10,000 × g for 10 min. Nuclei containing the nuclear envelope and 5% Triton X-100 in PBS. The matrices were collected with a pipette and diluted in CSK 50 till a final concentration of approximately 2 mg/ml.

**Gel Electrophoresis and Immunoblotting**—The protein concentration was determined according to Peterson (31). Nuclear fractions were resuspended in sample buffer (60 mM Tris, 10% glycerol (v/v), 4% sodium dithiothreitol, 80 mM sodium dodecyl sulfate, pH 6.8). The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 5-15% gradient gel. Proteins were stained with Coomassie Brilliant Blue or blotted onto nitrocellulose (Schleicher and Schuell, Dassel, F. R. G.) as described previously (32). Nitrocellulose sheets were blocked with 0.3% Protilar (Nutricia, Zoetermeer, The Netherlands) in PBS for 1 h at 37°C and incubated with the primary antibody diluted in 0.03% Protilar in PBS for 1 h at 37°C. The sheets were washed in the same buffer and subsequently incubated with secondary antibodies conjugated to alkaline phosphatase. The sheets were subsequently washed with 0.03% Protilar/PBS and detected with 5-bromo-4-chloro-3-indolyl phosphate.

**Phosphoinositide Metabolism and Nuclear Matrices**

**Lipid Kinase Assays—**Since no or few endogenous lipids were found in the nuclear matrix preparations, lipid kinase activities were measured in a total volume of 100 μl containing 50 mM Tris/HCl (pH 7.4), 10 mM MgCl2, 60 μM ATP, indicated concentrations of exogenous lipids, and nuclear proteins. PtdIns, PtdIns(4)P, and DAG were dried under an N2 stream independently or mixed together as indicated with double amount of phosphatidylinserine and vesicles were made by mixing 50 μl Tris/HCl (pH 7.4) and sonicated for 2 min on ice using an MSE sonicator at maximum output. Freeze fracture studies showed that the vesicles are unilamellar, having a diameter between 30 and 300 nm. The reaction was started by adding 10 μl of [γ-32P]ATP and carried out at room temperature under shaking for 10 min as described previously (64). Under all circumstances, assays were linear with protein concentration and with time. After stopping the reaction by adding 400 μl of mixture of 0.1 M fluoride/methanol (v/v), lipids were immediately extracted following the modified method of Bligh and Dyer (35, 36). Phosphoinositides and phosphatidic acid were separated by thin layer Chromatography on silicic acid/neutral silica gel-Chromato graph 90/70/20 (as a solvent (37). After autoradiography 32P-labeled lipids were detected and scraped off and radioactivity was quantified. Results are presented as mean of three independent experiments.

**Lipid Analysis—**Identification of the phosphoinositides was performed using HPLC technique according to Auger et al. (38). Briefly, lipids were deacylated from the total lipid extract and injected in a 4.6 mm × 100 mm Partisephe SAX column (Whatman International, Maidstone, U. K.). Radioactivity eluting from the column was monitored and quantified as reported previously (13). Authentic tritiated PtdIns, PtdIns(4)P, PtdIns(4,5)P2, as well as [32P] orthophosphate were used as standards for the identification of the phosphoinositides.

**Assay of Phospholipase C Against Exogenous Substrate—**PLC activity associated to the nuclear matrix was detected using [3H]PtdIns(4,5)P2 (Amersham International, Amersham, U. K.) as exogenous substrate. [3H]PtdIns(4,5)P2 (50,000 dpm, 1 nmol/assay) were dried under an N2 stream and dissolved in 5 mM sodium cholate, the reaction was performed at 30°C during 10 min as previously described (39). Results are presented as mean of three independent experiments.

**RESULTS**

**Isolation of Nuclei—**Membrane depleted nuclei from mouse NIH 3T3 fibroblasts were isolated as described under Experimental Procedures. Purity of these nuclei was analyzed using different enzyme markers as LDH activity as cytoskeletal marker, 5'-nucleotidase as plasma membrane marker, and nuclear matrix marker, and adenylate kinase as nuclear marker. As plasma membrane marker, and cytokeratin 8 (RCK 102) were a generous gift from F. C. S. Ramaekers (University of Limburg, The Netherlands). The antibodies directed against the internal matrix protein p160 (AM 88) and against tubulin from Sera Lab Ltd. (Sussex, U. K.) were obtained from Sigma.
antimycin A-insensitive NADH-cytochrome c reductase as marker for the endoplasmic reticulum. LDH activity in isolated nuclei was 0.38 ± 0.04% of LDH activity in total cell homogenates. Nuclear 5'-nucleotidase activity was 0.9 ± 0.4% of the activity of total cell homogenates and nuclear activity of the antimycin A-insensitive NADH-cytochrome c reductase was below detection level. These results are in accordance with data presented by Cocco et al. (11) and Masmoudi et al. (22).

Second, nuclear fractions were analyzed for contamination by cytoskeletal components. This is especially important since several enzymes from the phosphoinositide metabolism were found to be associated to the cytoskeleton (34). The cytoskeleton of NIH 3T3 cells consists of actin filaments, microtubuli, and type III intermediate filaments (vimentin) (40, 41). As shown in Fig. 1 (lane N), membrane-depleted nuclei from NIH 3T3 cells contain nuclear proteins such as lamin A and C and p160, an internal matrix protein (42). These nuclei are contaminated by actin and vimentin while tubulin is absent. Nuclear matrices (Fig. 1, lane M) were obtained from nuclei treated with a mixture of DNase I and RNase A and incubated with ammonium sulfate (24). Under these circumstances more than 95% of cellular proteins and more than 98% of the nucleic acids are removed (42). The effect of this extraction on protein composition is shown in Fig. 1. In the Coomassie Blue-stained gel a different staining pattern is observed after extraction. By Western blotting (Fig. 1), we show that the nuclear proteins lamin A and C and p160 are concentrated by this extraction. However, the cytoskeletal contamination remains attached to these nuclear matrices.

To avoid any contribution of this contamination in our measurements we decided to investigate nuclei from another cell type in parallel. In addition, by isolating nuclei in the absence of Triton X-100 we could check the effect of this detergent on a possible reallocation of the kinases during nuclei isolation. For this purpose we chose rat liver cells which have a cytoskeleton composed of actin filaments, microtubuli, and a different set of intermediate filaments, namely type I and II (keratin 18 and 8, respectively) (43); nuclei from rat liver cells were isolated in the absence of Triton X-100 as described by Izaurralde et al. (29). Membrane-depleted nuclei were subsequently obtained by an incubation of the nuclei in CSK100 containing 1% Triton X-100 for 7 min. As shown in Fig. 1, nuclear preparations from rat liver cells were devoid of actin and tubulin contamination, but intermediate filaments (keratins) were still present. We were not able to remove these intermediate filaments from the nuclei, suggesting a strong interaction between intermediate filaments and the nuclear matrix, which is in agreement with previous studies (23, 24). By comparing the results obtained in both cell types we may avoid a contribution of both cytoskeletal contamination and isolation procedure on our measurements.

Lipid Kinase Activities in Isolated Nuclei—The presence of phosphoinositide kinases and DAG kinase was analyzed in membrane-depleted nuclei as well as in nuclear matrices. No or little signal on TLC plates was obtained in the absence of exogenous lipids (data not shown). Incubation of lipid membrane-free nuclei isolated from NIH 3T3 fibroblasts with exogenous lipid vesicles containing PtdIns, PtdIns(4)P, and DAG in the presence of [γ-32P]ATP resulted in the formation of 3P-labeled PtdInsP, PtdInsP, and PtdOH (Fig. 2, lane N). In addition, the nuclear matrix was still able to phosphorylate the exogenous lipids (Fig. 2, lane M). Similar results were obtained for rat liver nuclear preparations (not shown).

The fraction of nuclear located activities of PtdIns kinase, PtdInsP kinase, and DAG kinase was determined by comparing enzyme activities in nuclei and total cell homogenates. Kinase activities in nuclei isolated from NIH 3T3 cells was 3.1 ± 0.8% for the PtdIns kinase, 13.7 ± 2.1% for the PtdInsP kinase, and 9.6 ± 2.2% for the DAG kinase. These results confirm that PtdIns kinase, PtdInsP kinase, and DAG kinase are present in membrane-free nuclei and in nuclear matrices (44).

Evidence for Differential Location of Lipid Kinases in the Nuclear Matrix—The nuclear matrix can be separated biochemically into the internal matrix (InM) and the peripheral matrix (PeM) by treatment with a reducing agent, like dithiothreitol (16, 17). The protein content of nuclear fractions obtained in this way was 1.9 ± 0.2% and 2.5 ± 0.1% of total cellular protein for the peripheral and internal nuclear matrix.
respectively. Purity of these fractions was analyzed by Western blotting, using lamin A and C as a marker for the peripheral matrix and p160 as a marker for the internal matrix (42). As shown in Fig. 3 (lanes InM), the internal matrix preparation from both cell lines is not contaminated by lamins. On the other hand, the peripheral matrix preparation (Fig. 3, lanes PeM) is poorly (rat liver) or not (NIH 3T3) contaminated by internal matrix proteins. The intermediate filament subunits are only present in the peripheral matrix (vimentin in NIH 3T3 cells and keratin in rat liver nuclei). The actin contamination was found in both nuclear fractions of NIH 3T3 cells and is absent in rat liver preparations.

Subsequently, the presence of different lipid kinase activities was examined in peripheral and internal nuclear matrices. As shown in Fig. 4 (panel A), when PtdIns and PtdIns(4)P were incubated as exogenous lipids in the presence of [γ-32P]ATP and the intact nuclear matrices (lane M) both PtdIns kinase and PtdInsP kinase activities were detected as in Fig. 2 (lane M). However, when the peripheral matrix (PeM) was incubated under the same conditions only PtdIns kinase activity was detected. When the internal matrix preparation was incubated in the presence of PtdIns and PtdIns(4)P (Fig. 4A, lane InM) only 32P-labeled PtdInsP could be detected, indicating the presence of PtdInsP kinase in this fraction. PtdIns kinase activity was virtually absent in the internal matrix. The appearance of PtdOH (Fig. 4A, lane InM) is only possible if PtdInsP2 was hydrolyzed by a PLC to DAG immediately following its synthesis, and DAG phosphorylated to PtdOH by a DAG kinase. The presence of DAG kinase was confirmed as shown in Fig. 4B. Indeed, when the internal matrix preparation was incubated in the presence of DAG and [γ-32P]ATP (lane InM) we could detect a clear DAG kinase activity producing the PtdOH while a weak DAG-kinase activity was found in peripheral matrix preparations.

**FIG. 4. Formation of phosphorylated lipids by isolated nuclear matrices from NIH 3T3 cells.** 50 μg of proteins from intact nuclear matrices (lane M), internal nuclear matrix (lane InM), and peripheral nuclear matrix (lane PeM) obtained from NIH 3T3 cells were incubated in the presence of exogenous lipid vesicles of PtdIns (50 μM), PtdIns(4)P (50 μM) (in panel A) or DAG (50 μM), PS (100 μM) (in panel B) and [γ-32P]ATP. The 32P-phosphorylated lipids were localized by autoradiography; the positions of mentioned standards after migration on TLC are indicated (O, origin).

<table>
<thead>
<tr>
<th></th>
<th>PtdIns-kinase</th>
<th>PtdInsP-kinase</th>
<th>DAG-kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact nuclear matrices (M)</td>
<td>2.40 ± 0.10</td>
<td>1.50 ± 0.40</td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td>Internal nuclear matrix (InM)</td>
<td>0.08 ± 0.07</td>
<td>5.00 ± 0.70</td>
<td>4.50 ± 1.00</td>
</tr>
<tr>
<td>Peripheral nuclear matrix (PeM)</td>
<td>6.10 ± 1.50</td>
<td>0.30 ± 0.08</td>
<td>0.40 ± 0.15</td>
</tr>
<tr>
<td>InM/PeM</td>
<td>0.03</td>
<td>3.35</td>
<td>4.10</td>
</tr>
<tr>
<td>PeM/PeM</td>
<td>2.55</td>
<td>0.20</td>
<td>0.35</td>
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In conclusion, as indicated in Table I, the specific activity of PtdIns kinase is clearly enriched in the peripheral matrix while only traces of this activity were measured in the internal matrix from nuclei of NIH 3T3 cells. On the other hand PtdInsP kinase, DAG kinase, and PLC activities were strongly enriched in the internal matrix (Tables I and II).

In order to determine whether these enzymes are indeed associated to an internal nuclear matrix structure we dialyzed the internal nuclear matrix preparation to remove the salt and dithiothreitol. After several hours, internal matrix proteins start to aggregate and these aggregates were pelleted by centrifugation. 65.6 ± 9% of the internal matrix proteins were found in the pellet. Also 64 ± 6% and 72.5 ± 9% of the

**FIG. 3. Analysis of nuclear matrix proteins.** Nuclear matrices (M), internal matrix (InM), and peripheral matrix (PeM) were obtained as described under "Experimental Procedures." 20 μg of protein was separated on a 5-15% gradient SDS-PAGE (CB, Coomassie Blue-stained gel). For immunoblotting, matrices from 109 NIH 3T3 nuclei and 6 x 106 matrices from rat liver were probed for the presence of different proteins as indicated.
Phosphoinositide Metabolism and Nuclear Matrices

TABLE II

| PLC specific activities (pmol x min⁻¹ x mg⁻¹) in nuclear matrix fractions |
|-----------------------------|------------------|-------------------|
|                             | NIH 3T3          | Internal nuclear matrix |
| Intact nuclear matrices     | 6.5 ± 1.5        | 18.5 ± 6.8         |
| Peripheral nuclear matrix   | 1.8 ± 0.4        |                   |

PtdIns kinase and DAG kinase activity, respectively, were measured in this pellet, suggesting a tight association of these enzymes to internal nuclear matrix structures.

Identification of Phosphoinositide Kinases—For a precise identification of the type of phosphoinositide kinases which are bound to the nuclear matrix, separation of the products of these lipid kinases by HPLC is necessary. Moreover, we used this technique to compare the nuclear matrix associated phosphoinositide kinases from NIH 3T3 cells and rat liver cells. This comparison is necessary to investigate a possible influence of the contaminating cytoskeletal components present in these nuclear preparations as described above.

As clearly shown in Fig. 5, lane M, the lipid kinases present in intact nuclear matrix from NIH 3T3 cells are PtdIns 4-kinase and PtdIns(4)P 5-kinase. In peripheral nuclear matrix (PeM) only PtdIns 4-kinase activity was detected while in internal nuclear matrix preparations (InM) only PtdIns(4)P 5-kinase and DAG kinase were present. These results confirm unambiguously the results obtained by TLC (Fig. 4).

In intact rat liver nuclear matrices, a similar profile was observed (Fig. 5, lane M), with, however, the presence of traces of PtdIns 3-kinase activity. The internal nuclear matrix from rat liver (Fig. 5, lane InM) contains, exactly as in internal matrix from NIH 3T3 cells, PtdIns(4)P 5-kinase activity. The peak of PtdOH indicates the presence of DAG kinase and suggests also the presence of PLC activity. Indeed, we could measure a specific activity of 11 ± 5 pmol/min/mg for PLC in this internal matrix preparation. Since we failed to obtain pure peripheral matrix preparations from rat liver (contamination by internal matrix; see Fig. 2, lane PeM) the HPLC profile obtained indicated the presence of a main PtdIns 4-kinase activity besides a PtdIns(4)P 5-kinase activity due to the internal matrix contamination (not shown).

In conclusion, similar results were obtained in isolated nuclear matrices from NIH 3T3 and rat liver cells allowing us to suggest a differential location of these lipid kinases in the nucleus.

DISCUSSION

We demonstrate in this paper that the nuclear matrix of mammalian cells contains phosphoinositide kinase, DAG kinase, and PLC activities. Although previous studies have reported the presence of these enzymes in the nucleus (10, 11), the identification of the phosphoinositide kinases as well as the precise location of these enzymes were unknown. Using HPLC techniques we show that the PtdIns 4-kinase and PtdIns(4)P 5-kinase are present in nuclear matrices of two mammalian cell types, mouse NIH 3T3 fibroblasts and rat liver cells. On the other hand we provide biochemical evidence for a differential location of the enzymes involved in the phosphoinositide metabolism in the nucleus. PtdIns 4-kinase is found to be associated to the peripheral matrix, while the internal matrix was virtually devoid of this activity.

PtdIns(4)P 5-kinase and DAG kinase were found to be associated preferentially to internal nuclear matrix structures. PLC activity was also clearly enriched in the internal nuclear matrix. Similar results were found in both NIH 3T3 and rat liver nuclei preparations, suggesting that this differential location is not cell type-dependent.

An important aspect concerns the possible contamination of cytosolic proteins in our nuclear preparations. Nuclei were isolated in the presence (NIH 3T3 fibroblasts) and in the absence of Triton X-100 (rat liver cells). Nuclear preparations obtained following these procedures were checked for cytoplasmic contamination with several marker enzymes: lactate dehydrogenase, 5'-nucleotidase, and antimycin A-insensitive NADH-cytochrome c reductase, and activities of these enzymes were shown to be less than 1% in the nuclear fraction when compared with the activity in total cell homogenates. The obtained results with nuclei from NIH 3T3 fibroblasts and rat liver cells were similar which excludes a possible effect of Triton X-100 on the location of PI kinases, DAG kinase, and PLC in the nucleus. This is in agreement with earlier studies reported by Cocco et al. (11), who could not observe a significant difference in PI kinase activities in membrane-containing and membrane-depleted nuclei.

In contrast with the above mentioned enzyme markers for nuclei purity, contamination of isolated nuclei with cytoskeletal elements is more difficult to avoid (23, 24). In our study we have tried to remove cytoskeletal elements from these nuclei by applying several different protocols. We were not able to remove all intermediate filaments from nuclei or from the peripheral matrix, which suggests a tight association of the intermediate filaments with the lamina-pore complex. Similar results were obtained previously (23, 24, 28) and with binding studies using 32P-labeled vimentin, it has been shown that vimentin binds specifically to lamin B (27). Contamination with actin was only observed in 3T3 nuclei, and it is absent in rat liver nuclei. Tubulin, however, is absent in nuclei isolations of both 3T3 cells and rat liver cells. This result was expected, since at 4°C microtubules are depolymerized and the tubulins then partition as free cytosolic proteins. To avoid a possible contribution of both the isolation procedure and cytoskeletal contamination we have compared nuclei from two different cell types containing different cytoskeletal elements, which are isolated following different isolation protocols.

In this paper we have also addressed the question why PI and DAG kinases are found in the different subnuclear fractions. The PtdIns 4-kinase precipitates together with the peripheral matrix, indicating that this kinase binds to this fraction. The PtdIns(4)P 5-kinase and the DAG kinase are solubilized by a reduction of the intact nuclear matrix with dithiothreitol. After the removal of the salt and dithiothreitol by dialysis, the internal matrix proteins start to aggregate. By centrifugation we showed that both kinases coprecipitate with the internal matrix fraction. This implies that the observed differential location of PI and DAG kinases in the nucleus is caused by differential binding affinities to proteins in either the peripheral or the internal matrix.

An interesting aspect concerns the regulatory mechanism of the phosphoinositide metabolism in the nucleus. In this respect it is of interest to note that it was recently reported that the PtdIns concentration in the nucleus from rat liver is suboptimal for the PtdIns kinase activity. An intervention of PtdIns-transport protein providing the PtdIns to the nucleus was suggested (45). Since we found the PtdIns 4-kinase in the peripheral nuclear matrix, one can expect a rapid phosphorylation of the PtdIns provided by PtdIns-transport protein to
FIG. 5. Identification of the phosphoinositide kinases by HPLC technique and comparison between NIH 3T3 and rat liver nuclear preparations. 50 µg of proteins from intact nuclear matrices (M), internal nuclear matrix (InM), or peripheral nuclear matrix (PeM) obtained from NIH 3T3 cells or rat liver were incubated in the presence of exogenous lipid vesicles of PtdIns (50 µM), PtdIns(4)P (50 µM), and [γ-32P] ATP. Lipids were extracted, deacylated, and separated on a HPLC column as indicated under "Experimental Procedures." Abbreviation: GroP, glycerophospho-

PtdIns(4)P in this matrix and a subsequent production of PtdIns(4,5)P2 in the internal matrix by PtdIns(4)P 5-kinase. This compound may be hydrolyzed to inositol trisphosphate and DAG by PLC, which appears to be very active in the internal matrix. DAG may modulate protein kinase C activity which has been shown previously to be present in the nucleus (46–48) or may be phosphorylated to PtdOH by the DAG kinase which is also preferentially located in the internal nuclear matrix.

The role of these compounds in the nucleus is still unknown, but the apparent compartmentalization of the enzymes responsible for their formation suggest a specific and important role. It has been shown in vitro that PtdInsP and inositol 1,4-bisphosphate activate DNA polymerase α (5), which is one example of the potential effect of phosphoinositides and inositol phosphates on nucleic acid metabolism. Moreover, changes in the phosphoinositide metabolism in lipid membrane-free nuclei has also been reported upon growth factor treatment or dependent on the state of differentiation of cells (11, 12). Thus, a relationship may exist between the so-called signal transduction and the phosphoinositide cycle in the nucleus. Two coordinated mechanisms may be involved: the substrate availability and the modulation of the enzyme activities. Taken together, these results indicate that the phosphoinositide metabolism which is present in the nucleus may be strictly regulated during the cell cycle. Further studies will be necessary to elucidate the exact role of phosphoinositide metabolism in the nucleus and its modulation by different factors implicated in cell proliferation.

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