

Lipid phosphorylation in isolated rat liver nuclei

Synthesis of polyphosphoinositides at subnuclear level

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Isolated rat liver nuclei and subnuclear fractions synthesize polyphosphoinositides *in vitro* in a mode dependent on the presence of nuclear membrane, detergent and exogenous substrates. The nuclear membrane is not essential as a source of lipid kinases, since the addition of exogenous phosphatidylinositol or phosphatidylinositol monophosphate to reaction mixtures lacking membranes restores the synthesis of phosphatidylinositol mono- and bisphosphate, respectively. Inositol phosphorylation is best accomplished by high-salt extracted nuclei and pre-detergent lamina. These data suggest that the nucleus, and especially the nuclear periphery, is a cell compartment in which polyphosphoinositide synthesis occurs; this might be related to the progression of phosphatidylinositol metabolism-dependent signals to the genetic apparatus.

Polyphosphoinositide; Polyphosphoinositide kinase; Nuclear matrix; (Rat liver)

1. INTRODUCTION

Synthesis of PI occurs essentially in the endoplasmic reticulum, while its phosphorylation appears to be more widely distributed throughout the cell, since PI-kinase and PIP-kinase have been described in association with the cell membrane and other organelles [1]. As to the nuclear envelope, it has been shown that it is able to incorporate ³²P into PIP, PIP₂ and PA [2], suggesting that the cell nucleus accomplishes at least part of the inositol lipid cycle, which, at the plasma membrane level, is involved in the signal transduction system utilized by a number of stimuli such as hormones, growth factors and neurotransmitters [3,4].

More recently, we have demonstrated that isolated nuclei synthesize the same lipids in membrane-free systems in a manner dependent on both differentiation and proliferative events [5-7].

This evidence supports previous data on the metabolic and structural role of nuclear lipids, which can function as regulatory molecules involved in the control of nuclear function [8-12], and suggests that the cell nucleus is a site of lipid phosphorylation, not necessarily involving enzymes and substrates located on the nuclear membrane.

In this paper we report a study of the fractionation of rat liver nuclei devised to evaluate the lipid phosphorylation in different nuclear compartments, and report evidence for the presence of PI-kinase and PIP-kinase in membrane-free subnuclear fractions such as nuclear matrix and lamina.

2. MATERIALS AND METHODS

2.1. Isolation of nuclei and subnuclear fractions

As previously described [13], the procedure included purification of the nuclei from male adult Wistar rats (250-300 g) by low and high density sucrose buffer centrifugation; extraction involved the following steps: endogenous digestion at 37°C for 45 min, 2 extractions in 0.2 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM Tris-HCl, pH 7.4 (low-salt buffer, LS); 2 extractions in 2 M NaCl, 0.2 mM MgCl₂, 1 mM

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PMSF, 10 mM Tris-HCl, pH 7.4 (high-salt buffer, HS); one wash in LS containing 0.4% Triton X-100.

The nuclear lamina was purified with the same procedure employed for the nuclear matrix except that all buffers contained 5 mM dithiothreitol (DTT) and the isolated nuclei were digested with DNase I (14 U/mg DNA) and RNase A (80 μ g/mg DNA) for 30 min in ice [14]. Aliquots of pre-detergent lamina were saved and the nuclear lamina was obtained with one final extraction in LS containing 0.4% Triton X-100. Membrane-depleted nuclei were isolated with sucrose buffers containing 0.2% Triton X-100. The protein and DNA content of the fractions derived from nuclei was determined by flow cytometry on samples stained with fluorescein isothiocyanate and ethidium bromide, respectively [15].

The preparations of nuclei and subnuclear fractions were checked for purity by ultrastructural and enzymological analysis [16,17], and characterized by polyacrylamide gel electrophoresis on 0.1% SDS denaturing gels according to [18]. Protein assay was as described in [19].

2.2. Lipid phosphorylation

Liver homogenate, whole nuclei and subnuclear fractions (200 μ g protein) were preincubated for 10 min at 30°C in the presence or absence of 0.05% Triton X-100, and of 100 μ M PI or PIP (Sigma), and then incubated for 5 min with 20 mM $MgCl_2$, 10 mM β -mercaptoethanol (β ME), 1 mM vanadate, 100 μ M ATP and 1 μ Ci [γ - ^{32}P]ATP (5000 Ci/mmol, Amersham) in a final volume of 200 μ l.

The reaction was terminated by addition of 4 ml chloroform/methanol/HCl (200:100:0.75, v/v) and the phosphoinositides were extracted according to Shaikh and Palmer [20]. Labelled lipids and known internal standards were analyzed on 1% oxalate-impregnated silica gel plates (Merck) developed with chloroform/methanol/water/ammonia (45:35:8:2, v/v). The plates were autoradiographed on Kodak X-Omat S film, before exposure to iodine, and the spots were scraped off the plates and counted by liquid scintillation.

3. RESULTS AND DISCUSSION

The absence of contaminating plasma membranes or cytoplasmic structures in nuclear and subnuclear fractions was morphologically assessed, and negligible amounts of 5'-nucleotidase and glucose-6-phosphatase were found, in agreement with previous data [21]. The presence of detergent during purification ensured the removal of nuclear membrane, as demonstrated by ultrastructural analysis and by the assay of electron transport components, such as NADH-cytochrome *c* and NADH-ferricyanide reductase [16]. In addition, nuclei isolated with the same procedure were shown to be free of redistributed cytoplasmic lipids labelled with tritiated glycerol and inositol, confirming that extranuclear membranes do not adventitiously stick to the nuclei during homogenization

and subsequent purification [5]. The composition of the residual fractions after extraction as described in section 2 reveals a decrease of DNA and proteins with the purification steps (table 1). Analysis on SDS-polyacrylamide gels indicates that many proteins are progressively lost from nuclei to matrix and lamina. After the high salt extraction, histones were virtually removed, while lamin A, B and C were among the major components of the nuclear matrix, and largely predominated in lamina (fig.1).

The phosphorylation profiles show PIP, PIP₂, PA, and some additional bands. One of these, according to co-migration with authentic internal standard, was tentatively identified as lyso-PIP.

Liquid scintillation counting of the spots (table 2) indicates that the recovery of labelled PIP and PIP₂ under control conditions was larger in membrane-depleted nuclei than in whole nuclei. As expected, the incorporation levels were lower than in total homogenate, but not as low as the levels predicted on the basis of the evidence that most of the lipid kinase activities are located in the plasma membrane [2]. Furthermore, the specific activity of PI-kinase increased with purification, reaching, in pre-detergent matrix and lamina, maximum levels well above those of total homogenate. The same feature characterized the PIP-kinase, except that it dropped in pre-detergent lamina. The PI-kinase activity displayed by the corresponding membrane-free structures, nuclear matrix and lamina, con-

Table 1

Flow cytometric analysis of rat liver nuclei and subnuclear fractions after two-color staining with EB (ethidium bromide) and FITC (fluorescein isothiocyanate), staining nuclei acids and proteins, respectively

Step	EB	FITC
Whole nuclei	100	100
LS fraction	15	58
HS fraction	6	20
Nuclear matrix	3	14
Lamina	0.04	3

Red and green fluorescence were analyzed with a FACSTAR flow cytometer (Becton Dickinson) operating at 300 mW ($\lambda_{\text{exc}} = 488$ nm). Data represent the percent of fluorescence signals remaining after each of the purification steps that lead from nuclei to nuclear matrix and lamina. Staining was with 50 μ g/ml EB and 0.01 μ g/ml FITC for 30 min. Mean of 4 determinations, with SD < 8%

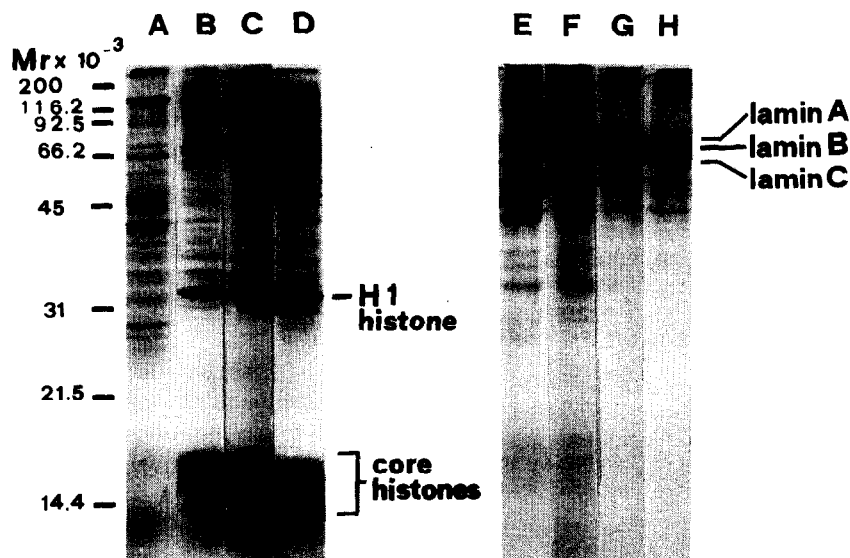


Fig.1. Electrophoretic characterization of rat liver fractions. Proteins were analyzed on 0.1% SDS/12.5% polyacrylamide gels and stained with Coomassie R-250. Approx. 50 μ g proteins were loaded on each lane. A, total homogenate; B, whole nuclei; C, membrane-depleted nuclei; D, LS fraction; E, HS fraction; F, nuclear matrix; G, pre-detergent lamina; H, lamina.

firmed the tendency to increase, while PIP-kinase was not detectable. As to the diacylglycerol kinase (DG-kinase), it seemed predominantly membrane-bound, since it was reduced in membrane-depleted nuclei in comparison to whole nuclei, and absent in matrix and lamina once the membrane was removed.

The addition of 0.05% Triton X-100 in the assay produced activation of PI-kinase and PIP-kinase in membrane-containing structures, except for the pre-detergent lamina, and a general inhibition of DG-kinase, in agreement with the role of non-ionic detergents in facilitating or inhibiting expression of maximum activity [22].

If exogenous PI or PIP was included in the assay, the recovery of PIP and PIP₂ respectively was increased. This was more evident in membrane-depleted nuclei, nuclear matrix and lamina, where it reached a 10–20-fold increase. In the presence of PIP and detergent the recovery of labelled PIP was enhanced in membrane-depleted nuclei, matrix and lamina, consistent with a phosphomonoesterase-catalysed breakdown of PIP to PI which could be then converted to PIP.

The behaviour of total homogenate, whole nuclei and the LS fraction, suggests that phosphomonoesterases, phosphodiesterases and possibly

inhibitors of the lipid kinases can seriously hamper the recovery of phosphorylated lipids.

Furthermore, total homogenate and whole nuclei are much less sensitive to the addition of exogenous substrates than the heavily extracted fractions. This might be due either to the fact that the concentrations of endogenous substrates are sufficient to saturate the enzymes, or to reduction of apparent incorporation by lipid breakdown. In nuclear matrix and lamina, the lipid kinase activities are readily modulated, clearly depending on the presence of the membrane and on the additions. In these fractions, it seems that synthetic processes exceed degradation, since PI-kinase and PIP-kinase are able to express their activities. Even though LS, HS and pre-detergent lamina fractions showed an activity much higher than the corresponding membrane-depleted fractions, the addition of exogenous substrates indicates that lipid kinases are, remarkably, present also after removal of the membrane. This implies that the nuclear membrane is able to provide both enzymes and substrates for PI metabolism, but its importance seems to be more critical for substrates than for enzymes.

The resistance of lipid kinases to digestion, extensive extractions and detergent, supports the con-

Table 2

In vitro synthesis of PA, PIP and PIP₂ by rat liver nuclei and subnuclear fractions incubated with [γ -³²P]ATP

		Additions			
		None (%)	Triton (%)	PI Triton (%)	PIP Triton (%)
Total homogenate	PA	456 (3.8)	361 (2.9)	340 (3.1)	415 (3.7)
	PIP	10914 (90.9)	11585 (95.3)	10274 (94.5)	10301 (93.4)
	PIP ₂	638 (5.3)	214 (1.8)	259 (2.4)	316 (2.9)
Whole nuclei	PA	249 (12.3)	- -	66 (0.6)	129 (1.0)
	PIP	1572 (77.7)	10711 (98.6)	10441 (98.3)	10494 (81.4)
	PIP ₂	201 (9.9)	147 (1.4)	118 (1.1)	2264 (17.6)
Membrane-depleted nuclei	PA	83 (1.0)	- -	271 (1.6)	117 (1.4)
	PIP	8050 (94.6)	890 (89.4)	15365 (92.8)	5848 (72.2)
	PIP ₂	376 (4.4)	105 (10.6)	932 (5.6)	2133 (26.4)
LS fraction	PA	401 (5.0)	311 (0.7)	202 (0.9)	617 (1.3)
	PIP	7025 (87.1)	42005 (97.2)	22748 (96.3)	42896 (89.8)
	PIP ₂	635 (7.9)	919 (2.1)	672 (2.8)	4263 (8.9)
HS fraction	PA	774 (3.1)	623 (0.6)	216 (0.2)	721 (1.1)
	PIP	22107 (89.2)	84989 (88.3)	88002 (86.0)	57662 (90.4)
	PIP ₂	1893 (7.7)	10621 (11.1)	14125 (13.8)	5394 (8.5)
Nuclear matrix	PA	- -	- -	- -	- -
	PIP	199 (100.0)	995 (100.0)	23420 (98.8)	2411 (54.7)
	PIP ₂	- -	- -	274 (1.2)	2000 (45.3)
Pre-detergend lamina	PA	517 (1.9)	- -	- -	110 (1.2)
	PIP	25724 (95.0)	7676 (98.0)	7688 (96.3)	9016 (95.6)
	PIP ₂	829 (3.1)	158 (2.0)	294 (3.7)	302 (3.2)
Lamina	PA	- -	- -	- -	- -
	PIP	1342 (100.0)	1166 (100.0)	13410 (98.1)	7272 (95.5)
	PIP ₂	- -	- -	256 (1.9)	340 (4.5)

Data are expressed as cpm/mg protein, the percentages being given in parentheses. Where indicated, additions are: 0.05% Triton, 100 μ M PI and PIP. Mean values from at least 5 experiments. Standard deviations below 12%

tention that they are actually nuclear in origin and tightly bound to nuclear structures. Under control conditions, the nuclear matrix-membrane complex (HS fraction) and the lamina-membrane complex (pre-detergent lamina) show by far the highest PI-kinase and PIP-kinase activity. This is consistent with the hypothesis that the periphery of the nucleus is a crucial site where key regulatory events take place. Considering the signal transduction system in which phosphorylation of inositol lipids and PKC-dependent phosphorylation of proteins are coupled, our data might correlate with the reported presence or translocation of PKC in

nuclei and subnuclear fractions [16,23,24], and the PKC directed phosphorylation of lamin B in lymphocytes and HL 60 cells [25,26]. Additional modulatory events are likely to take place also in the inner nuclear compartment, reliant equally on the presence and accumulation of inositol-derived molecules [27], phosphoinositide kinase [5,6] and PKC [16,28-30].

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