RESEARCH COMMUNICATION

Phosphoinositide signalling enzymes in rat liver nuclei: phosphoinositidase C isoform $\beta 1$ is specifically, but not predominantly, located in the nucleus

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The presence of phosphoinositide-mobilizing enzymes has been investigated in purified rat liver nuclei by radiolabelling and by probing with antibodies. A Ca^{2+} -activated phosphoinositidase C (PIC) is present and was shown immunologically to be the $\beta 1$ isoform. No γ - or δ -PIC was found. However, only 5% of the total $\beta 1$ -PIC isoform is nuclear, with the majority being cytosolic. $\text{G}\alpha_q$ and $\text{G}\alpha_{11}$, the suggested physiological activators of $\beta 1$ -PIC,

were not present. A $\text{PtdIns}4\text{P}$ 5-kinase is also present, which immunologically is shown to be the C isoform. All of these nuclear inositide enzymes still remained after the removal of the nuclear envelope with Triton X-100, consistent with the concept of an intranuclear inositide cycle [Divecha, Banfic and Irvine (1991) *EMBO. J.* **10**, 3207–3214].

INTRODUCTION

The activation of the phosphoinositide cycle located in the plasma membrane, in response to hormone binding, has been extensively investigated and many of the aspects of its regulation are now understood (Nishizuka, 1988; Berridge and Irvine, 1989). Recently, however, evidence has accumulated suggesting the presence of a similar cycle in the nucleus and we know very little about the enzymology, regulation and function of this cycle [reviewed by Irvine and Divecha (1992)]. Initial evidence for the occurrence of the cycle in the nucleus was presented by Smith and Wells (1983), who demonstrated that the nuclear membrane contained all the elements for the production of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$, the substrates for phosphoinositidase C (PIC). This was taken further by Cocco et al. (1988, 1989), who demonstrated that some aspect of this metabolism could be changed by insulin-like growth factor (IGF-1) binding at the plasma membrane of a Swiss 3T3 cell. In a detailed study of the mass levels of the phosphoinositides and of diacylglycerol (DAG) in the nucleus, we were able to demonstrate that stimulation of Swiss 3T3 cells with IGF-1 led to a decrease in the mass of $\text{PtdIns}4\text{P}$ and $\text{PtdIns}(4,5)\text{P}_2$ with a concomitant increase in the nuclear DAG (Divecha et al., 1991). These results are compatible with the notion that there is present in the nucleus a PIC which can be stimulated by activation of the IGF-1 receptor at the plasma membrane. A number of workers have demonstrated the presence of PIC activity in the nucleus (Payrastra et al., 1992; Kuriki et al., 1992; Martelli et al., 1992). Moreover, Martelli et al. (1992) suggested that the $\beta 1$ -isoform of PIC is exclusively localized within the nucleus in Swiss 3T3 cells. This initial observation has been followed up by a report by Mazzoni et al. (1992) showing the same observation on PC 12 cells. It has recently been demonstrated (H. Banfic, N. Divecha and R. F. Irvine, unpublished work) that 20 h after partial hepatectomy there is a rise in nuclear DAG levels and a translocation of protein kinase C to the nucleus, which corresponds with the beginning of S phase as seen by Fabrikant (1968), suggesting that

the nuclear inositide cycle is present in rat liver nuclei and is subject to regulation.

Rat liver nuclei are easily prepared in a pure form with no detergent-washing steps, so they offer an ideal system to begin a study of nuclear inositide enzymes. Here we have undertaken an immunological study to look at the presence of different isoforms of PIC in rat liver nuclei. We demonstrated, in agreement with Martelli et al. (1992), that only the $\beta 1$ -isoform of PIC was present in the nucleus, the other isoforms, $\gamma 1$ and $\delta 1$, being present solely in the cytosolic and membrane fraction. However, in contrast with the results obtained by Martelli et al. (1992) in Swiss 3T3 cells, the majority of $\beta 1$ -PIC was localized in the cytosolic and membrane fractions of rat liver. We also present evidence for the presence of $\text{PtdIns}4\text{P}$ 5-kinase C (Divecha et al., 1992), and absence of $\text{G}\alpha_q/\text{G}\alpha_{11}$ in the same nuclei.

MATERIALS AND METHODS

All rats used in this study were male Wistar rats weighing approx. 250 g. Rats were killed by cervical dislocation and all following procedures during the isolation of the nuclei were carried out at 4 °C. Monoclonal antibodies to the $\gamma 1$ and the $\delta 1$ isoforms were as described (Suh et al., 1988), and polyclonal antibodies were prepared against purified $\beta 1$ -PIC (Ryu et al., 1987). The polyclonal antibody to the $\beta 1$ enzyme is totally specific and does not recognize $\beta 2$ or $\beta 3$.

Nuclei preparation

The procedure utilized was a modification of the method of Blobel and Potter (1966) and is described in detail below. The livers from two male rats were collected on ice and 14 g (wet wt.) was homogenized in 28 ml of solution B (10 mM Hepes, pH 7.5/5 mM MgCl_2 /25 mM KCl) using a power-driven pestle. Solution C (10 mM Hepes, pH 7.5/2 mM MgCl_2 /2.4 M sucrose) (4.9 ml) was added to give a final concentration of sucrose of 0.25 M. A sample (7.5 ml) of this solution was then mixed with 15 ml of solution D (10 mM Hepes, pH 7.5/2 mM MgCl_2 /2.3 M

Abbreviations used: PIC, phosphoinositidase C; DAG, diacylglycerol; TBS, Tris-buffered saline; IGF, insulin-like growth factor; NPB, nuclear phosphorylation buffer (2 × = 100 mM Hepes, pH 7.4/10 mM MgCl_2 /200 mM NaCl).

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sucrose) and mixed by inversion to give a final sucrose concentration of 1.62 M. A 7.5 ml cushion of solution D was then layered below this using a syringe and was spun at 106000 *g* for 30 min at 4 °C in an SW 27 rotor. The pellet at the bottom of the lower cushion was considered to be the nuclear pellet and was washed twice with solution A (10 mM Hepes, pH 7.5/2 mM MgCl₂/0.25 M sucrose) and finally resuspended in 5 ml of this solution. The upper phase and the interphase of the two sucrose cushions were pooled, diluted to 336 ml with water, and re-spun at 9000 *g* in 50 ml tubes in a 6×50 Europa fixed-angle rotor to pellet the microsomal and membrane fractions. The supernatant from this fraction was defined as the liver cytosol in these experiments. The pellet was resuspended in approx. 5 ml of solution A and was considered as the microsomal fraction. Protein estimation were carried out on all of these fractions using the Bio-Rad protein assay. From two rat livers there was a yield of approx. 20 mg of nuclear protein, 50 mg of microsomal protein and 900 mg of cytosolic protein.

Triton extraction of nuclei was carried out by washing nuclei for 15 min in solution A containing 0.4% Triton X-100; the nuclei were then pelleted, washed once with solution A and resuspended in their original volume. Nuclei designated as control nuclei went through the same procedure as the Triton-washed samples, except that the Triton X-100 was omitted.

Nuclear lipid labelling

Nuclei (100 µg of protein) were put into 50 µl of double-strength nuclear phosphorylation buffer (NPB; 2× buffer = 100 mM Hepes, pH 7.4/10 mM MgCl₂/200 mM NaCl) and made up to a volume of 95 µl. The reaction was started by the addition of 5 µl of NPB containing 2 mM ATP and 10 µCi of [γ -³²P]ATP. The reactions were stopped by the addition of 750 µl of chloroform/methanol (1:2, v/v) followed by the addition of 250 µl of chloroform and 250 µl of 2.4 M HCl. The phases were separated by brief centrifugation and washed twice with theoretical upper phase. The lipid was dried down, spotted on to t.l.c. plates and then developed with chloroform/methanol/water/conc. ammonia (45:35:8:2, by vol.). After drying, spots were located by autoradiography and compared with standards. Quantification was carried out by removing the spots by scraping and counting by liquid scintillation.

PIC assay

Nuclei (approx. 40 µg of protein) were incubated in 10 mM Hepes, 2 mM MgCl₂, 0.14 M NaCl and 0.25 M sucrose and the reaction was started by the addition of 3 nmol of PtdIns(4,5)P₂ containing 20000 c.p.m. of PtdIns(4,5)P₂ (labelled at locant 5 with ³²P) in a total volume of 200 µl. After 5 min the reaction was stopped by the addition of 1 ml of chloroform/methanol (1:1, v/v) followed by 250 µl of 2.4 M HCl. After brief centrifugation in a Microcentaur, the top phase was removed and counted by Cerenkov radiation. For the acid molybdate assay the upper phase was dried overnight, resuspended in 500 µl of perchloric acid (PCA; 10%, v/v), centrifuged (Microcentaur, full speed) and 100 µl of ammonium molybdate (5%, w/v) added. This was followed by the addition of 1.2 ml of isobutyl alcohol/toluene (1:1, v/v), and vortexing followed by centrifugation (Microcentaur, full speed). The upper phase, which is the organic phase, contains the inorganic phosphate, while the lower phase contains the Ins(1,4,5)P₃. In a typical analysis approx. 98% of the counts were found to be in the phase containing the

Ins(1,4,5)P₃, illustrating the absence of any phosphomonoesterase activity.

SDS/PAGE and immunoblotting

Proteins for electrophoresis were prepared such that the concentration of each sample was 50 µg/25 µl in sample loading buffer (Laemmli, 1970). In the case of the nuclei the samples (approx. 1 mg) were incubated on ice for 20 min with 1 µg of DNAase I (Sigma) to break down the DNA. Electrophoresis was carried out using a Bio-Rad Minigel apparatus at the acrylamide concentration stated in the Figure legends. After electrophoresis the proteins were transferred to nitrocellulose using a Bio-Rad wet-blotting system according to the method of Towbin et al. (1979). After blocking the nitrocellulose in 20 mM Tris/140 mM NaCl/0.05% (v/v) Tween 20/4% (w/v) dried milk (Marvel), the blots were incubated with primary antibody at the concentration stated in the legends for 2 h, followed by washing in the above buffer and incubation with the appropriate secondary antibody conjugated to horseradish peroxidase. Visualization was carried out using the ECL kit (Amersham).

RESULTS

Smith and Wells (1983) first reported that nuclei had the ability to synthesize PtdIns4P and PtdIns(4,5)P₂ in the presence of ATP. Rat liver nuclei, prepared as described in the Materials and methods section of this paper, yielded nuclei which were extremely pure. In terms of lactate dehydrogenase activity the nuclei contained approx. 0.37% of the cytosolic activity, in good agreement with Payraastre et al. (1992). We were also unable to see any Ins(1,4,5)P₃ phosphatase or kinase activity (results not shown). We then looked at the ability of the nuclei to incorporate [γ -³²P]ATP into PtdIns4P, PtdIns(4,5)P₂ and phosphatidic acid before and after washing with Triton X-100 to remove the nuclear membrane. As seen before, the nuclei would synthesize these lipids when the nuclear membrane was present (Cocco et al., 1987). However, after washing with Triton no incorporation into inositides was found (Figure 1). Addition of exogenous DAG to Triton-washed nuclei yielded phosphatidic acid. However, addition of either PtdIns or PtdIns4P did not yield any labelled PtdIns4P or PtdIns(4,5)P₂ (Figure 1). The incorporation of label into PtdIns(4,5)P₂ could be markedly inhibited by low concentrations of Triton, so it is difficult to tell whether the absence of incorporation into Triton-washed nuclei is due to inhibition or removal of the PtdIns4P 5-kinase. To resolve this, we used Western blotting with a monoclonal antibody to the C isoform of PtdIns4P 5-kinase (Divecha et al., 1992). We have previously purified this isoform and have raised monoclonal antibodies to it. The enzyme was present in the cytosol of the liver and was also present in the nuclei. However, washing with Triton did not remove any of this enzyme (Figure 2). So, the inability of the enzyme to make PtdIns(4,5)P₂ using exogenous PtdIns4P may be due to either the presence of Triton or possibly the removal of some other factor required for the activity of the kinase. We do not believe that this is a problem of substrate availability, as sonication of the Triton-washed nuclei before addition of the lipid did not yield any labelled PtdIns(4,5)P₂; sonication does not apparently inhibit inositide kinases, as incorporation into non-Triton-washed nuclei was not affected by this treatment (results not shown).

Measurement of the activity of PIC in the nuclei, before and after washing, was carried out using high-specific-radioactivity ³²P-labelled PtdIns(4,5)P₂ generated by the phosphorylation of PtdIns4P using a purified PtdIns4P 5-kinase (N. Divecha, un-

Figure 1 The nuclear inositide cycle in rat liver nuclei

Nuclei (100 μ g) were incubated with [32 P]ATP for 5 min before (Cont) or after washing with Triton X-100 (Triton wash). Lipids in the form of sonicated vesicles were added as stated in the Figure to either the control or the Triton-washed nuclei. As can be seen, washing with Triton removes the ability of the nuclei to incorporate label into the lipids. Re-addition of either PtdIns or PtdIns4P (3 nmol) did not restore the ability of the nuclei to synthesize these lipids. The addition of DAG, however, does lead to the synthesis of phosphatidic acid.

Figure 2 PtdIns4P 5-kinase isoform C (Divecha et al., 1992) is present in rat liver nuclei

Cytosol (lane 1) or nuclei before (lanes 2 and 3) or after (lanes 4 and 5) washing with Triton X-100 were subjected to SDS/PAGE using 10% (w/v) acrylamide, transferred to nitrocellulose and probed with a mixture of monoclonal antibodies (diluted 1:5) specific to isoform C (see Divecha et al., 1992). The preparation and characterization of these antibodies will be described elsewhere. The enzyme was present in the cytosol of rat liver as well as the nuclei. Triton washing of the nuclei, although leading to the inhibition of incorporation of [32 P]ATP into PtdIns(4,5) P_2 (see Figure 1), did not remove this enzyme. Lane 6 contains purified PtdIns4P 5-kinase C isoform.

published work). Acid molybdate extraction of the water-soluble radioactivity demonstrated that the counts were not present as inorganic phosphate, suggesting that under the conditions of the assay there was no PtdIns(4,5) P_2 phosphomonoesterase activity. Thus any water-soluble radioactivity was a measure of the PIC activity. There was no change in the amount of activity recovered from control nuclei as compared with nuclei washed with Triton (Table 1), and the activity corresponded to approx. 2 nmol/min per mg of nuclear protein. Both the PIC activities from either

Table 1 Effect of Ca^{2+} and Triton washing on nuclear PIC activity

Nuclei were either kept as controls or were washed with 0.4% Triton X-100 to remove the nuclear membrane. Washing with Triton X-100 did not lead to any significant decrease in PIC activity. The Table also shows the effect of the free Ca^{2+} concentration on the PIC activity from control nuclei. The same result was also seen with nuclei after washing with Triton X-100 (results not shown).

Treatment	Ins(1,4,5) P_3 produced (nmol/min per mg of nuclear protein)
Control nuclei	2.1
Triton-washed nuclei	1.9
[Ca^{2+}]	
5 nM	0
50 nM	0.05
10 μ M	1.6
100 μ M	1.2
500 μ M	0.6
1 mM	0.25

Figure 3 The β 1-isoform of PIC is the only isoform present in the nucleus, but is not exclusively localized there

Total homogenates (1), cytosolic (2), particulate (3) and nuclear fractions before (4) and after (5) Triton extraction were subjected to SDS/PAGE using 7% (w/v) [anti-(PIC γ) or (PIC β) antibody] or 10% (w/v) [anti-(PIC δ) or anti-(Gq $_{\alpha/11}$) antibodies] acrylamide gels and transferred to nitrocellulose. These were then probed with a polyclonal antibody specific for β 1-PIC (1:5000) (a) or monoclonal antibodies specific for the γ 1 (1:1000) (b) or the δ 1 (1:1000) (c) isoform. As can be seen only the β 1-isoform was present in the nucleus (a, lanes 4 and 5). Although the δ isoform of PIC has been shown to be an 85 kDa protein, the polypeptides recognized in these blots were approx. 45 kDa. Lane 6 was partially purified δ -PIC. In (d) the fractions were as above but were probed with an antibody derived against a peptide common to Gq $_{\alpha}$ and Gq $_{11}$ (diluted 1:1000).

control or from Triton-washed nuclei showed the same activation by Ca^{2+} (showing a stimulatory effect at 10 μ M; see Table 1) and were both stimulated 10-fold by deoxycholate (0.1%). Thus the PIC activity present in rat liver nuclei appears to be an integral component of the nucleus, in agreement with the results obtained from Payastre et al. (1992). We were also interested in which PIC isoform was present in the nucleus. Immunoblotting data with antibodies specific for the different PIC isoforms showed that β 1, δ 1 and γ 1 were all present in the total homogenates from rat liver (Figure 3). The immunoreactive band recognized by the δ 1-isoform antibody appeared to be a doublet of molecular mass 45 kDa, although the positive control (partially purified δ 1 isoform) showed up as an 85 kDa protein. This could not be

prevented even in the presence of protease inhibitors and EGTA. Indeed, recent observations have suggested that this isoform is susceptible to cleavage during the isolation of tissue fractions (S.-G. Rhee, unpublished work). PIC $\gamma 1$ was found to be solely localized to the cytosol, while the $\beta 1$ and the $\delta 1$ isoforms were both found in the cytosolic and particulate fractions. When nuclei were probed with the various antibodies, only the $\beta 1$ isoform was found in the nuclear fraction. Washing with Triton did not affect the amount of $\beta 1$ -PIC present, suggesting that it was probably bound to some internal matrix as suggested by Payraastre et al. (1992). As $\beta 1$ -PIC was the only isoform present in the nuclear fraction, and because this isoform of PIC has been shown to be stimulated by either G_q or G_{q11} (Taylor et al., 1991; Smrcka et al., 1991), we used an antibody directed against a peptide sequence common to both of these proteins to screen our different fractions. As can be seen, the antibody recognized a 43 kDa protein present in the total homogenate and in the particulate fraction (the high-molecular-mass protein recognized by the antibody has been seen before and appears to show a non-specific cross-reactivity with the antibody; G. Milligan and M. Wakelam, personal communication). No immunoreactivity was found in the cytosolic fraction, suggesting that the protein is associated with a membrane fraction, as has been suggested previously. No immunoreactive protein was found in the nuclear fraction (Figure 3d).

DISCUSSION

In this study we have demonstrated the presence of two of the isoenzymes possibly involved in the nuclear inositide cycle. The first is PtdIns4P 5-kinase C, an isoform that was initially purified from human erythrocytes (Ling et al., 1989; Bazen et al., 1990) and later from bovine brain (Divecha et al., 1992). We have produced a bank of monoclonal antibodies against this isoform of the kinase and have used these to screen the different fractions of liver. This is the first definition of a specific PtdIns4P 5-kinase isoform in the nucleus.

Although the presence of PIC has been documented in nuclear fractions (Payraastre et al., 1992; Kuriki et al., 1992; Martelli et al., 1992) there is still some dispute over the intracellular location of the various isoforms. In Swiss 3T3 cells Martelli et al. (1992) have suggested that the $\beta 1$ -PIC is exclusively localized in the nuclear fraction even before stimulation with IGF-1, an agonist which stimulated nuclear inositide metabolism (Divecha et al., 1992.) However, many of the seven membrane-domain receptors are thought to stimulate the plasma membrane inositide cycle via a heterotrimeric G protein, and the G_q family of G proteins have been shown to stimulate only the β -PICs without any effect on the other isoforms (Taylor et al., 1991; Smrcka et al., 1991; Lee et al., 1992). Furthermore, antisera prepared against specific epitopes of the $G_{q\alpha}$ and $G_{q11\alpha}$ were found to inhibit the GTPase activity, as well as blocking the activation of PIC by specific ligands such as thromboxane in membrane preparations (Shenker et al., 1991), and so it appears to be unlikely that this isoform of the enzyme would be localized solely to the nucleus. In this study we have used rat liver as a suitable tissue to study the nuclear distribution of the various PICs, as it is easy to obtain pure nuclear fractions in large yield in the absence of any detergent, so minimizing the redistribution of proteins during the isolation procedure. Although the $\beta 1$, $\delta 1$ and $\gamma 1$ isoforms were present

in the total homogenates, only the $\beta 1$ -isoform was clearly present in the nucleus. In the blots shown (Figure 3), equivalent protein was loaded, and in terms of the initial liver homogenate (see the Materials and methods section) the amount of nuclear $\beta 1$ -PIC therefore represents only approx. 2% of the total $\beta 1$ -PIC, with the cytosolic component representing approx. 90%. This is in contrast with the $G_{q\alpha}/G_{q11\alpha}$ where the microsomal/membrane fraction contains approx. 100% of the immunoreactivity with essentially no immunoreactivity in the nucleus. The fact that only the $\beta 1$ -PIC is represented in the nucleus and that neither of the other two isoforms of PIC, nor another wholly particulate protein, $G_{q\alpha}/G_{q11\alpha}$, are represented in the nucleus (Figure 3), is a convincing argument against the presence of the $\beta 1$ -isoform being just a contamination artefact. The difference between the results obtained by Martelli et al. (1992) and the results presented in this study is difficult to reconcile, but may be due to the methods of preparation or the differences in cell type. However, we think that an enzyme that is probably used by a variety of plasma membrane receptors is unlikely to be wholly associated with the nucleus.

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