Identification of high levels of protein phosphatase-1 in rat liver nuclei

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Rat liver nuclei contain a protein phosphatase that is indistinguishable from the catalytic subunit of protein phosphatase-1 in its molecular mass, sensitivity to inhibitor-1 and inhibitor-2 and specificity for the β-subunit of phosphorylase kinase. This activity is not bound to the outer nuclear membrane, but located within the nucleus. The average level of protein phosphatase-1 activity in nuclei is at least 5-fold higher than its average extranuclear concentration.

Nucleus *Protein phosphatase* *Metabolic regulation* *Protein phosphorylation* *(Rat liver)*

1. INTRODUCTION

Four protein phosphatase catalytic (C)-subunits, termed 1, 2A, 2B and 2C, have been identified in mammalian tissues that dephosphorylate a variety of regulatory proteins. Protein phosphatase-1 (PP-1) is distinguished by its sensitivity to the thermostable proteins inhibitor-1 (I-1) and inhibitor-2 (I-2) and specificity for the β-subunit of phosphorylase kinase, whereas the type-2 protein phosphatases are unaffected by I-1 and I-2 and preferentially dephosphorylate the α-subunit of phosphorylase kinase [1,2].

When homogenates of mammalian tissues are centrifuged for 60 min at 100000 × *g*, at least 70% of the directly measurable PP-1 activity is recovered in the 100000 × *g* pellet, whereas PP-2A, PP-2B and PP-2C are largely found in the 100000 × *g* supernatant (Chisholm, S., McGowan, C. and Cohen, P., unpublished). These observations suggest that the active forms of PP-1 are associated with organelles and membranes, and there is increasing evidence that supports this view. For example, a substantial proportion of the PP-1 in skeletal muscle is bound to glycogen [3,4], and PP-1 is the major phosphatase associated with ribosomes in reticulocytes [5] and with postsynaptic densities in the brain [6]. The glycogen-bound form of PP-1 has been purified from rabbit skeletal muscle and shown to consist of the 37 kDa C-subunit complexed to a G-subunit responsible for anchoring the phosphatase to glycogen [7]. This raises the possibility that PP-1 may be complexed to a variety of 'targetting' proteins that direct the C-subunit to particular locations within cells [8].

Here, we demonstrate the presence of surprisingly high levels of PP-1 in liver nuclei.

2. MATERIALS AND METHODS

2.1. *Preparation of nuclei and nuclear extracts*

Nuclei were isolated at 0–4°C from fed adult female Wistar rats (3–5 months old) as described by Blobel and Potter [9], except that 0.1% (v/v) 2-mercaptoethanol and the proteinase inhibitors phenylmethylsulphonyl fluoride (1 mM), benzamidine (1 mM), pepstatin (0.01 mM) and leupeptin (0.01 mM) were included. The nuclear pellets were resuspended in 50 mM Tris-Cl, pH 7.5 (25°C), 25 mM KCl, 5 mM $MgCl_2$, 0.1% (v/v) 2-mercaptoethanol, 0.1 mM phenylmethylsul-

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phonyl fluoride and 1 mM benzamidine (buffer A) plus 250 mM sucrose, using 2.5 ml/g liver. To remove the outer nuclear membranes, resuspended nuclei were made 0.5% (w/v) in Triton X-100, rehomogenised using three strokes of a glass-teflon homogeniser and recentrifuged for 5 min at 800 × *g*. The supernatant was removed and the pellets resuspended in buffer A + 10 mM EDTA (2.5 ml/g liver). In order to lyse the nuclei, 0.33 vols of 50 mM Tris-Cl, pH 7.5 (25°C), 0.1% (v/v) 2-mercaptoethanol, 2 M NaCl were added, and the suspension stirred for 30 min. The nuclear lysates were then centrifuged for 30 min at 240000 × *g* to pellet the chromatin, and the supernatant, termed nuclear extract, decanted. Contamination of the nuclear lysate by cytoplasm, mitochondria, microsomes and lysosomes was negligible as judged by assays with standard marker enzymes.

2.2. *^{32}P-labelled protein substrates (10^6 cpm/nmol)*

Phosphorylase *a* (glycogen phosphorylase, EC 2.4.1.11; 1 mol phosphate/mol 97 kDa subunit) was phosphorylated using phosphorylase kinase (EC 2.7.1.38). [^{32}P]Phosphorylase kinase (1.4–1.6 mol phosphate/$\alpha\beta\gamma\delta$ unit) containing equal amounts of ^{32}P radioactivity in the α- and β-subunits was labelled with cyclic AMP-dependent protein kinase (EC 2.7.1.37). Procedures for phosphorylating each protein and for freeing ^{32}P-labelled substrates from [γ-^{32}P]ATP were described in [10].

2.3. *Protein phosphatase assays*

Protein phosphatases-1 and -2A were assayed at 30°C in an incubation mixture (0.03 ml) containing 50 mM Tris-Cl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 5 mM caffeine, bovine serum albumin (1.0 mg/ml) and phosphorylase *a* (1.0 mg/ml, 10.3 μM). Reactions were initiated with phosphorylase *a* after preincubating the other components for 5 min at 30°C. Assays were carried out for 10 min and terminated and analysed as in [11]. Release of radioactivity was restricted to <30% to ensure that rates of dephosphorylation were linear with respect to time. Assays were performed in duplicate and control incubations included in which protein phosphatase was replaced by buffer. These values were subtracted from those obtained in the presence of phosphatase. One unit of activity (U) was that amount of enzyme which catalysed the dephosphorylation of 1.0 μmol phosphorylase *a* per min. Liver homogenates were assayed at a final dilution of 1:600 and nuclear lysates and extracts at a final dilution of at least 1:60.

Protein phosphatases 2B (PP-2B) and 2C (PP-2C) were assayed using [^{32}P]phosphorylase kinase as substrate. PP-2C was assayed in an identical manner to that described for PP-1 and PP-2A, except that magnesium acetate (20 mM) was included. PP-2C was the activity in the absence of Mg^{2+} subtracted from that in the presence of Mg^{2+}. PP-2B was assayed in the presence of 0.1 μM calmodulin and 0.88 mM $CaCl_2$–1.0 mM EGTA to give a free Ca^{2+} concentration of 3 μM [4,12]. PP-2B was the activity in the presence of EGTA subtracted from activity in the presence of 3 μM Ca^{2+}. Inhibitor-2 (0.1 μM) was included in assays of PP-2B and PP-2C to inactivate PP-1.

2.4. *Other procedures*

Inhibitor-1 [13] and inhibitor-2 [14] were purified from rabbit skeletal muscle. Protein was measured by the procedure of Bradford [15] using bovine serum albumin ($A^{1\%}_{280\,nm}$ = 6.5) as standard. DNA was assayed according to Labarca and Paigen [16], using calf thymus DNA as standard ($A^{1\%}_{260\,nm}$ = 200).

3. RESULTS

Phosphorylase phosphatase activity in nuclear lysates or extracts did not increase with dilution, provided that assays were carried out at a 60-fold final dilution or greater. The level of activity (12–15 mU/g liver) was about 10% of that present in the liver homogenates (table 1). However, DNA recovery averaged only 50% (table 1), indicating that phosphorylase phosphatase activity associated with nuclei is about 20% of the level present in the homogenate.

If the nuclei were assayed before lysis in 500 mM NaCl, little phosphorylase phosphatase activity was detected, suggesting that the activity was not bound to the outer surface of the nuclear membrane. This view was reinforced by experiments in which the nuclei were washed with Triton, a procedure which removes the outer nuclear mem-

Table 1

Recovery of phosphorylase phosphatase activity and DNA during isolation of rat liver nuclei

Fraction	Phosphorylase phosphatase		DNA recovery (%)
	mU/g liver	Specific activity (mU/mg)	
Homogenate	120 ± 4.7	0.97 ± 0.21	100
Triton X-100 wash	1.6 ± 1.5	2.0 ± 0.5	<5
Nuclear lysate	12.0 ± 2.4	9.7 ± 4.7	49 ± 17
Nuclear extract	14.4 ± 1.7	13.2 ± 5.2	<5

Values are given ± SD for 3 separate preparations

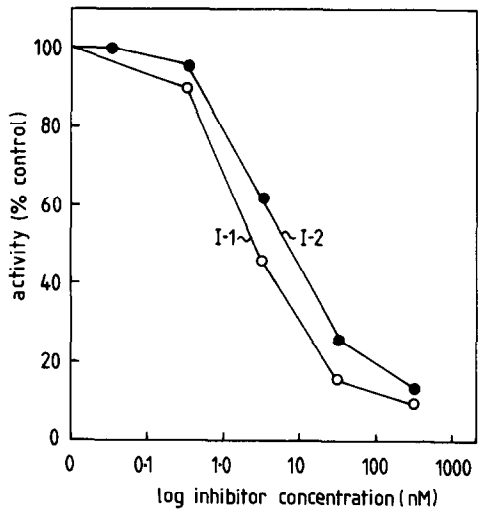

Fig.1. Effect of inhibitor-1 (I-1) and inhibitor-2 (I-2) on phosphorylase phosphatase activity in the nuclear extract. Assays were carried out as described in section 2, except that either I-1 (○) or I-2 (●) were included. Activities are expressed relative to control incubations in which the inhibitors were omitted.

brane, without causing nuclear lysis. Only 15% of the phosphorylase phosphatase activity released by lysis with 500 mM NaCl was extracted with Triton, demonstrating that the activity was located within the nucleus.

All the trichloroacetic acid-soluble ^{32}P radioactivity released from [^{32}P]phosphorylase *a* following incubation with the nuclear lysate could be complexed with molybdate and extracted into isobutanol:benzene (not shown). This established that the activity being measured was a phosphatase, and not a proteolytic activity releasing trichloroacetic acid-soluble ^{32}P-peptides.

Phosphorylase phosphatase activity in the nuclear lysates (not shown) or nuclear extracts (fig.1) could be inhibited almost completely by either I-1 (I_{50} = 2.5 nM) or I-2 (I_{50} = 6 nM), indicating that nearly all the directly measurable phosphorylase phosphatase activity was PP-1. This view was confirmed by experiments with [^{32}P]phosphorylase kinase, which showed that the nuclear extracts dephosphorylated the β-subunit much faster than the α-subunit (fig.2).

Approximately half of the phosphorylase phosphatase activity in liver homogenates is catalysed by PP-1 and half by PP-2A in the standard essay [4] and unpublished). The amount of PP-1 associated with nuclei is therefore about 40% of the PP-1 that is directly measurable in a liver homogenate. Since nuclei only occupy 6% of the volume of a hepatocyte [9], PP-1 activity within nuclei would appear to be ≃7-fold higher than the

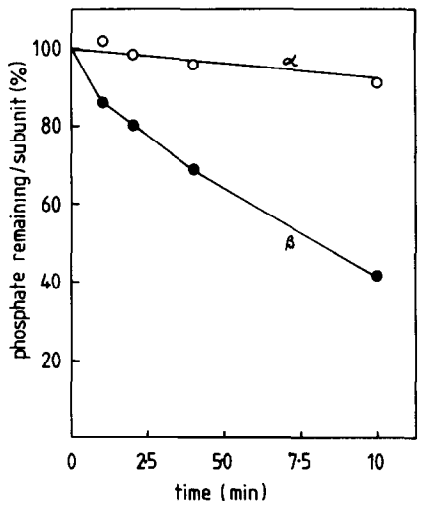

Fig.2. Selective dephosphorylation of the β-subunit of phosphorylase kinase by nuclear extract. Incubations were carried out using 0.35 mg/ml phosphorylase kinase as described in section 2. The amount of phosphate remaining in the α-subunit (○) and β-subunit (●) at various times was quantitated as in [1].

average extranuclear concentration. The specific activity of PP-1 in the nuclear lysates (10 mU/mg) is ≈20-fold higher than in the homogenates (table 1), assuming that half the homogenate activity is due to PP-1 and half to PP-2A.

PP-1 is the major protein phosphatase associated with hepatic glycogen and hepatic microsomes [18]. In these fractions, phosphorylase phosphatase activity due to PP-1 increases considerably with dilution. In the present work, phosphorylase phosphatase activity associated with glycogen and microsomes was ≈6 and ≈15 mU/g liver respectively, when assays were carried out at the highest possible dilutions. The amount of PP-1 within nuclei (12 mU/g liver without correction for DNA recovery) is therefore at least as high as the amount that is bound to glycogen or microsomes.

The free C-subunit of PP-1 is inhibited instantaneously by I-1 and I-2, whereas the higher molecular mass forms associated with glycogen and microsomes require 10–20 min preincubation with the inhibitors in order to achieve maximal inhibition [7,18]. Inhibition of nuclear PP-1 by I-1 and I-2 was not increased by preincubation, and

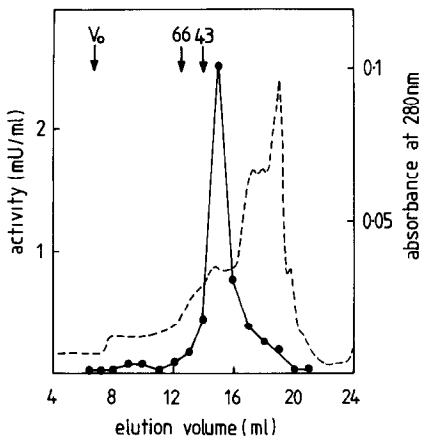

Fig.3. Gel filtration of nuclear extract on Superose-12. The sample (0.4 ml) was applied to the column (29 × 1 cm) equilibrated at ambient temperature in 50 mM Tris-Cl, pH 7.0 (25°C), 0.15 M NaCl, 0.1% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol. The flow rate was 0.9 ml/min and fractions of 1 ml were collected. Phosphatase activity is denoted by the closed circles and full line and absorbance at 280 nm by the broken line. The arrows show the positions of the void volume (V_0) and the marker proteins bovine serum albumin (66 kDa) and ovalbumin (43 kDa).

the I_{50} values were similar to those obtained with the pure C-subunit of PP-1 from rabbit skeletal muscle [7,18]. These observations suggested that PP-1 might be present in the nuclear extract as the free C-subunit. To test this idea, nuclear extracts were subjected to gel filtration on Superose 12. PP-1 activity was eluted as a single peak (apparent molecular mass ≈35 kDa) in the same position as the C-subunit of PP-1 (fig.3).

Nuclear lysates were also assayed for PP-2B and PP-2C using phosphorylase kinase as a substrate, as described in section 2. No Ca^{2+}/calmodulin-dependent (PP-2B) or Mg^{2+}-dependent (PP-2C) phosphorylase kinase phosphatase activity was detected.

4. DISCUSSION

Glucagon and insulin control the rate of transcription of a variety of hepatic enzymes. The effects of glucagon are mediated by cyclic AMP, and there is increasing evidence that the effects of this second messenger on transcription are mediated by cyclic AMP-dependent protein kinase.

Interaction of cyclic AMP with the regulatory (R)-subunit of cyclic AMP-dependent protein kinase, releases the active catalytic (C)-subunit which can then enter the nucleus. This has been reported in a number of cells, including rat hepatocytes exposed to glucagon [20]. Translocation of the C-subunit from a Golgi-associated location to the nucleus within minutes has been demonstrated by immunofluorescence studies in bovine epithelial (MDBK) cells following exposure to forskolin or dibutyryl cyclic AMP. By contrast, the type-II R-subunit remains associated with the Golgi complex in the presence or absence of forskolin [21]. In the pig kidney cell line LLC-PK1, agents that elevate cyclic AMP (calcitonin, forskolin) cause a 200-fold induction of plasminogen activator [22], but induction is decreased by 90% in mutant cells deficient in the major cyclic AMP-dependent protein kinase activity (Hemmings, B.A., personal communication).

The information which specifies accumulation of large proteins in the cell nucleus is encoded in a small region of the protein usually rich in lysine residues [23]. However, while the C-subunit of cyclic AMP-dependent protein kinase may enter

the nucleus by a facilitated transport mechanism, passive diffusion is also possible because it is small enough (40.5 kDa) to pass unhindered through the nuclear pores [24]. Alternatively, the hydrophobic myristyl group at its N-terminus [25] may facilitate diffusion across the nuclear membrane. However, unlike most nuclear proteins, translocation of the C-subunit is rapidly reversible, reassociation with the R-subunit being observable within 5 min of removing forskolin from MTBK cells [21].

In the present study, we have shown that rat liver nuclei contain high levels of a protein phosphatase activity that is indistinguishable from the C-subunit of PP-1 in its molecular mass, sensitivity to I-1 and I-2, and specificity for the β-subunit of phosphorylase kinase. Although detailed peptide mapping and primary structure analysis will be necessary to establish that the nuclear and cytosolic enzymes are products of the same gene, their identity will be assumed in the following discussion.

A major question is clearly how the C-subunit of PP-1 enters the nucleus. One possibility is that the C-subunit is complexed to an N-subunit which facilitates transport of the phosphatase across the nuclear membrane. However, since PP-1 was only detected in the nuclear extracts as the free C-subunit, one would have to argue that the putative N-subunit was degraded during preparation of the nuclear extracts, or that lysis of the nuclei in 500 mM NaCl dissociated the C- and N-subunits, the latter sedimenting with chromatin. Further work is necessary to establish whether the nuclear form of PP-1 ever exists as a complex with another protein. Alternatively, the C-subunit may be translocated to the nucleus in an analogous manner to the C-subunit of cyclic AMP-dependent protein kinase. Thus PP-1 may be excluded from the nucleus by its association with another protein, translocation only occurring following dissociation of the complex, perhaps in response to hormonal signals. The free C-subunit of PP-1 (37 kDa) is slightly smaller than the C-subunit of cyclic AMP-dependent protein kinase and may therefore be capable of entering the nucleus by passive diffusion. Interaction with a nuclear protein(s) may then allow it to accumulate in this organelle.

After this work had been completed, we learned that Jakes et al. [26] had also found that high concentrations of the PP-1 C-subunit are present in rat liver nuclei. In their experiments nuclei were lysed by sonication at low ionic strength. Under these conditions, most of the PP-1 was associated with chromatin, from which the free C-subunit could be released by extraction with either NaCl or treatment with DNase. Recently, Friedman [27] also showed that in nuclear extracts, protein phosphatase activity towards casein and nuclear phosphoproteins could be partially inhibited by I-2. Jakes et al. [26] have also demonstrated that nuclei contain an inactive form of PP-2A, whose activity is only expressed in the presence of basic proteins, such as histone H-1.

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