Inositol is a constituent of detergent-solubilized immunoaffinity-purified rat liver 5'-nucleotidase

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myo-Inositol analysis of detergent-solubilized immunoaffinity-purified rat liver 5'-nucleotidase showed the presence of 1 mol of *myo*-inositol/mol of enzyme monomer. This provides unequivocal evidence that the ectoenzyme 5'-nucleotidase is attached to liver membranes by a glycosyl-phosphatidylinositol lipid anchor.

INTRODUCTION

5'-Nucleotidase is a widely distributed intrinsic plasma membrane ectoenzyme with its catalytic active site on the extracellular surface of the plasma membrane [1, 2]. It can dephosphorylate all ribonucleoside 5'-monophosphates but its main activity is the hydrolysis of AMP, producing extracellular adenosine. There is evidence that ecto-5'-nucleotidase has a regulatory function in modulation of neurotransmitter release [3], in control of blood flow [4] and in the immune system [5]. The observation that ecto-5'-nucleotidase can be endocytosed and recycled to the cell surface [6–8] has led to speculation concerning a function inside the cell as well as interest in the mechanism of internalization and recycling.

Despite indirect evidence that 5'-nucleotidase interacts with the cytoskeleton, no positive evidence for a cytoplasmic portion of the molecule has been obtained [9,10]. In common with several intrinsic membrane proteins attached to the lipid bilayer via a glycosyl-phosphatidylinositol (GPI) lipid anchor [11–14], 5'-nucleotidase is released from membranes by the action of phosphatidylinositol-specific phospholipase C (PI-PLC). However, solubilization of 5'-nucleotidase by PI-PLC varies in extent and susceptibility between different tissues [15–19], and is often less than that of other GPI-linked proteins such as alkaline phosphatase.

If ecto-5'-nucleotidase is GPI-anchored to the plasma membrane, then detergent-solubilized purified enzyme should contain stoichiometric quantities of inositol. This hypothesis was tested by analysis of immunoaffinity-purified detergent-solubilized rat liver 5'-nucleotidase, which has been well studied previously and shown to be a homodimer of glycosylated subunits each of M_r 70 000 [20,21].

MATERIALS AND METHODS

In the present experiments, rat liver 5'-nucleotidase was purified to homogeneity by a modification of the previously published method [20]. The membranes pelleted after homogenization of the rat livers (62 500 $g_{\max}$ for 90 min) were solubilized with 2% (w/v) sulphobetaine 14 for 1 h at room temperature with a yield ranging from 80 to 93% in four separate preparations.

The soluble fraction after centrifugation (48 000 $g_{\max}$ for 30 min) was made 100 mM-NaCl and 1% (w/v) Triton X-100, and passed through a DE52 ion-exchange column equilibrated in 100 mM-Tris/85 mM-HCl/100 mM-NaCl/1 mM-MgCl₂/0.05% (w/v) Triton X-100/0.05% (w/v) sulphobetaine 14, and bound to a column of monoclonal antibody 5N-E5 [21] coupled to Sepharose 4B. 5'-Nucleotidase was eluted from the column with cold (< 4 °C) 50 mM-diethylamine/0.05% (w/v) Triton X-100/0.05% (w/v) sulphobetaine 14, and neutralized immediately with 0.1 vol. of 2 M-Tris/1.95 M-HCl/10 mM-MgCl₂. Final purification was achieved as described previously [20] using monoclonal antibody 5N-E5 coupled to cellulose, and gave a yield of 40% with a specific activity of 376 units/mg of protein when the protein was assayed by the technique of Lowry *et al.* [22], using bovine serum albumin as standard.

Samples of purified 5'-nucleotidase were used for amino acid compositional analysis to obtain an accurate protein measurement and for *myo*-inositol analysis using a modification of the method of Sherman *et al.* [23]. Samples were precipitated with 10% (w/v) trichloroacetic acid (final concentration) and the pelleted protein was washed with cold acetone containing 20 mM-HCl. Samples of the buffer in which the protein was dissolved were treated in the same way for blank controls. The samples (approx. 50 μ g of protein) for amino acid analysis were hydrolysed in 6 M-HCl/7 mM-mercaptoethanol for 24 h and 72 h, and analysed on an LKB 4400 amino acid analyser at the Department of Biochemistry, University of Cambridge. It was found that 50 μ g of protein as measured by the method of Lowry *et al.* [22] was equivalent to 21.5 μ g by amino acid composition. Samples for inositol analysis (10.75 μ g of protein) were mixed with 125 pmol of *scyllo*-inositol internal standard and hydrolysed for 24 h in 6 M-HCl at 110 °C. Following hydrolysis, HCl was removed by vacuum and repeated drying from water and the products were reacted with 20 μ l of Sigma-sil A (Sigma) for 2 h. Aliquots of 1 μ l were analysed by g.c.-m.s. using a Hewlett-Packard 5890-MSD system with a CPSi1 8CB (Chrompack) column (25 m $\times$ 0.32 mm) eluted with He at 2.5 ml/min. The temperature programme was from 140 °C (2 min) to 250 °C at 5 °C/min (held for 15 min). Inositol-TMS (trimethylsilyl) derivatives were detected using

Fig. 1. Selected ion monitoring g.c.-m.s. analysis of 5'-nucleotidase for inositol content

Total ion chromatograms (m/z 305 plus m/z 318) of TMS-derivatized acid hydrolysates of (a) 5'-nucleotidase and (b) a corresponding buffer blank. The *scyllo*-inositol peak is due to added internal standard.

electron impact ionization and selected ion monitoring [23] of the characteristic ions: m/z 305 ($\text{TMSOCH}=\text{C}(\text{OTMS})-\text{CH}=\text{OTMS}$)⁺ and m/z 318 ($\text{TMSO}=\text{CHCH}=\text{C}(\text{OTMS})\text{CHOTMS}$)⁺ (dwell time 100 ms). Relative response factors for the different inositol isomers were determined empirically by integration of the ion chromatograms due to hydrolysed standards.

RESULTS AND DISCUSSION

The g.c.-m.s. analysis (Fig. 1a) showed the presence of *myo*-inositol in the 5'-nucleotidase samples. A trace of *chiro*-inositol was also found, but this was not considered significant, since the buffer blank samples also contained a trace of this isomer (Fig. 1b). Analysis of a 5'-nucleotidase sample in the absence of a *scyllo*-inositol internal standard showed that this isomer is absent from 5'-nucleotidase (results not shown). Integration of the ion chromatograms gave a value of 196 pmol of *myo*-inositol in 10.7 µg of 5'-nucleotidase. Assuming an unglycosylated monomer M_r of 58 000 for 5'-nucleotidase [24,25], this indicates a content of 1.06 mol of *myo*-inositol/mol of 5'-nucleotidase monomer. Since the purification of 5'-nucleotidase used in the present study consistently achieved at least 75% yields at individual steps (and > 40% yield overall), it is likely that all rat liver ecto-5'-nucleotidase contains *myo*-inositol.

The demonstration of the presence of inositol in solubilized immunoaffinity-purified rat liver 5'-nucleotidase at a 1:1 ratio to the enzyme monomer provides unequivocal evidence that this enzyme is linked to the membrane by a GPI anchor. Whether the GPI anchor

plays a role in ecto-5'-nucleotidase function [12,26] or movement on membrane traffic pathways remains to be established.

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