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Identification of the human hepatic microsomal glucose-6-phosphatase enzyme

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The glucose-6-phosphatase enzyme protein of the human hepatic microsomal glucose-6-phosphatase system was identified as a 36.5 kDa polypeptide. The 36.5 kDa glucose-6-phosphatase enzyme protein was shown to be absent in the microsomes isolated from a patient previously diagnosed as having a type 1a glycogen storage disease.

Glucose-6-phosphatase; Glycogen storage disease; Phosphohydrolase; (Liver microsome)

1. INTRODUCTION

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) catalyzes the terminal steps of both gluconeogenesis and glycogenolysis [1]. The biological importance of the glucose-6-phosphatase enzyme in the control of blood glucose levels is emphasized by the debilitating effects of both its complete absence in patients with type 1a glycogen storage disease [2–4] and the abnormally high levels of glucose-6-phosphatase and hepatic glucose production in diabetes mellitus [1,5]. Nevertheless virtually nothing is known about the human hepatic microsomal glucose-6-phosphatase enzyme protein. We recently identified the rat hepatic microsomal glucose-6-phosphatase enzyme as a 36.5 kDa polypeptide by ^{32}P-labelling the phosphoryl-enzyme intermediate formed during steady-state hydrolysis of glucose 6-phosphate [6]. Unfortunately the technique failed to identify the human hepatic glucose-6-phosphatase enzyme [6]. The human enzyme is much more unstable than the rat enzyme [3,6–8] and was completely inactivated by the techniques used and no labelled phosphoprotein was formed. Earlier studies using a variety of techniques had reported molecular masses for the human hepatic glucose-6-phosphatase enzyme of 100, 63, 70 and 58–64 kDa [9–11]. It was therefore important for the human hepatic microsomal glucose-6-phosphatase enzyme to be unequivocally identified as a first step in the isolation of human DNA probes to allow prenatal diagnosis and carrier detection of type 1a glycogen storage disease.

Here we have used a modified ^{32}P-labelling technique and monospecific antibodies to the rat hepatic microsomal glucose-6-phosphatase enzyme to identify the polypeptide(s) which contain(s) the active site of the human hepatic microsomal glucose-6-phosphatase enzyme.

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2. MATERIALS AND METHODS

Biotinylated anti-sheep antibody, streptavidin-linked peroxidase complex, ^{32}P-labelled tetrasodium pyrophosphate and ^{32}P-labelled ATP (to prepare ^{32}P-labelled glucose 6-phosphate

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145 mCi/mmol) were purchased from Amersham (England). 4-Chloro-1-naphthol, glucose 6-phosphate (monosodium salt), mannose 6-phosphate (monosodium salt) were from Sigma (Poole, England). Sodium cacodylate also from Sigma was recrystallized from 95% ethanol [12]. The amphoteric detergent Empigen BB (alkyl dimethylamine betaine) was the kind gift of Albright and Wilson (Whitehaven, England). Lubrol 12A-9 was obtained from ICI Organics Division (Manchester, England). Nitrocellulose was obtained from Schleicher and Schuell (Dassel, FRG). All other chemicals were analytical reagent grade.

2.1. *Preparation and assays of hepatic microsomes*

Microsomes were prepared from fresh unfrozen human hepatic needle or small wedge biopsy samples [3] and livers of starved Wistar rats [13] and assayed for glucose-6-phosphatase activity as previously described [14]. The type 1a glycogen storage disease patient was a 3-month-old female child which was diagnosed on enzymatic analysis of a liver biopsy specimen. The glycogen content of the liver was 75 mg/g liver (upper limit of normal values is 40 mg/g liver). The activity of the glucose-6-phosphatase enzyme in microsomes isolated from the liver biopsy specimen was less than 0.01 μmols of inorganic phosphate released from glucose 6-phosphate/min per mg microsomal protein. The normal range of human hepatic glucose-6-phosphatase activity is 0.3–0.7 μmol/min per mg microsomal protein [3]. Protein concentrations were measured by the method of Peterson [15]. Ethical approval for this study was given by the Paediatric/Reproductive Medicine Ethics of Medical Research Subcommittee of Lothian Health Board and the Tayside Health Board Ethical Subcommittee.

2.2. *^{32}P-labelling of glucose-6-phosphatase*

The ^{32}P-labelling of rat and human microsomal glucose-6-phosphatase with ^{32}P-labelled glucose 6-phosphate and inorganic pyrophosphate was carried out exactly as in [6] except for two important changes: (i) prior to incubation the microsomes were not fully disrupted with Triton X-100, then centrifuged to remove detergent, but were disrupted with 0.5% lubrol 12-A9, as described in [5] and immediately added to the incubation without centrifugation; (ii) the incubation was carried out at 0°C for 30 s rather than 30°C for 20 s.

2.3. *SDS-PAGE, autoradiography and immunoblot analysis*

SDS-PAGE and autoradiography were carried out exactly as described in [6]. Immunoblot analysis of microsomal samples was carried out exactly as described in [16] using sheep antiserum previously shown to be monospecific for the rat hepatic microsomal glucose-6-phosphatase enzyme [6,17].

3. RESULTS AND DISCUSSION

The hepatic microsomal glucose-6-phosphatase is a multicomponent system comprised of the glucose-6-phosphatase enzyme with its active site situated at the luminal surface of the endoplasmic reticulum membrane, and a translocase T_1, which mediates the entry of glucose 6-phosphate into the luminal compartment. A second translocase T_2 mediates the equilibration of the product phosphate and the substrate pyrophosphate. The mechanism of glucose permeation across the membrane has been designated T_3 [3,7,16,18].

3.1. *^{32}P-labelling of the human microsomal glucose-6-phosphatase enzyme*

The phosphoryl-enzyme intermediate of the rat hepatic glucose-6-phosphatase system formed during steady-state hydrolysis of glucose 6-phosphate has recently been identified as a 36.5 kDa polypeptide [6]. Glucose 6-[^{32}P]phosphate also labelled a similar 36.5 kDa polypeptide in rabbit, sheep, mouse and guinea pig hepatic microsomes [6] but failed to label any polypeptides in human hepatic microsomes [6]. The glucose-6-phosphatase enzyme activity from human hepatic microsomes is known to be more labile than the hepatic enzymes from other species [3,7,8]. Therefore the most likely explanation for the failure to form a phosphoryl-enzyme intermediate in human liver was that the human enzyme had been inactivated by the techniques used. The only other protein of the glucose-6-phosphatase system that has been identified and purified is T_2 [16] and it has the same molecular mass in both rat and human liver [16]. However it could not be assumed that the human hepatic glucose-6-phosphatase enzyme was similar to the rat enzyme and had a 36.5 kDa polypeptide phosphoryl-enzyme intermediate, as several earlier papers had suggested much higher molecular masses for the human enzyme of 100, 63, 70 and 58–64 kDa [11].

We therefore studied the effect of each step in the ^{32}P-labelling method on the activity of the human hepatic enzyme. Two steps were found to cause inactivation and were modified as described in section 2. Lanes 3 and 4 of fig.1 show that using the modified procedures with ^{32}P-labelled glucose 6-phosphate a polypeptide was labelled in both rat and human microsomes the same result was obtained using ^{32}P-labelled pyrophosphate as substrate (not shown). Lanes 3 and 4 of fig.1 were overloaded in terms of microsomal protein to determine whether any minor bands were labelled with ^{32}P. The overloading of the gel and the presence of glycogen in the samples is the most likely cause of most of the nonspecific retention of ^{32}P at the origin (see [6] for a more detailed explanation) as it

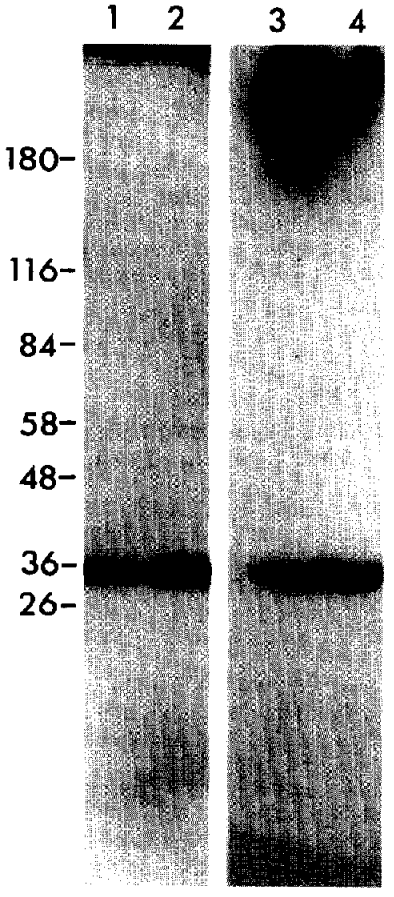

Fig.1. Immunoblot analysis and autoradiography of ^{32}P-labelled rat and human microsomes. Lanes 1–2 immunoblot analysis. Lane 1, ^{32}P-labelled fed rat hepatic microsomes (10 μg); lane 2, ^{32}P-labelled human hepatic microsomes (11.5 μg). Lanes 3 and 4, an autoradiograph. Lane 3, ^{32}P-labelled fed rat hepatic microsomes (100 μg); lane 4, ^{32}P-labelled human hepatic microsomes (104 μg).

was not ethical to starve human subjects prior to biopsy merely to lower the glycogen levels for this experiment.

3.2. *Immunodetection of the human hepatic glucose-6-phosphatase enzyme*

Previously it had been shown [17] that although the ^{32}P-labelled rat protein appears as one band on overloaded gels it can be resolved into a doublet on very lightly loaded gels [17]. Ten times less ^{32}P-labelled rat and human microsomal protein was subjected to immunoblot analysis to determine whether or not the human glucose-6-phosphatase enzyme protein was also a doublet. The antibody used was a sheep anti-rat hepatic microsomal glucose-6-phosphatase antiserum which had previously been shown to be monospecific for the 36.5 kDa polypeptide(s) in microsomes isolated from rat liver, kidney [6,17] and pancreatic islet cells [17]. Lanes 1 and 2 in fig.1 show that the antibody specifically cross-reacts with a 36.5 kDa polypeptide doublet in both rat and human microsomes. The identification of the immunoreactive polypeptide(s) in human microsomes as the glucose-6-phosphatase enzyme was confirmed (see fig.2, lane 3) by the complete absence of a cross-reacting protein in hepatic microsomes isolated from a patient previously diagnosed, by conventional assay methods [14], as having a type 1a glycogen storage disease. The protein identified here is clearly different from the 58–64 kDa polypeptide reported by Reczek and Villee [11] in both human liver and placenta. It has recently been

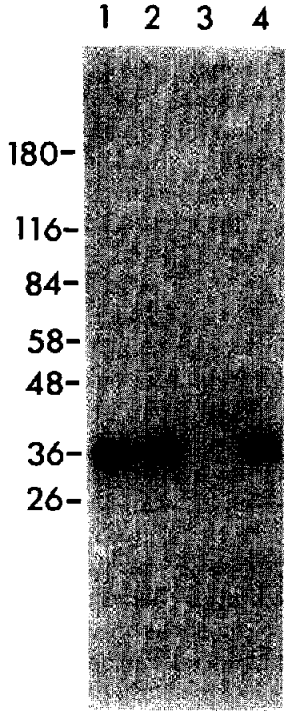

Fig.2. Immunoblot analysis of human hepatic microsomes. Lanes: 1, starved rat liver microsomes (10 μg); 2, control human hepatic microsomes (12 μg); 3, human hepatic microsomes from a patient with type 1a glycogen storage disease (12 μg); 4, human hepatic microsomes from a patient with type 1b glycogen storage disease (13 μg).

demonstrated [18] that human placenta does not contain the glucose-6-phosphatase enzyme system but that it has high levels of nonspecific phosphatase activity. The most probable explanation is therefore that Reczek and Villee [11] partially purified a nonspecific phosphatase from human liver obtained from autopsies, as the specific activity of glucose-6-phosphatase (0.01 μmol/min per mg) that they reported in frozen adult human hepatic microsomes was 50 times lower than the levels of glucose-6-phosphatase in fresh adult human liver [3].

3.3. *Conclusions*

The results of the experiments presented here establish the identity of the phosphohydrolase component of the human hepatic glucose-6-phosphatase system. This conclusion is supported by three independent lines of evidence. First, radioactivity from ^{32}P-labelled glucose 6-phosphate was specifically incorporated into the 36.5 kDa polypeptide(s) which correlate(s) exactly with the polypeptide(s) previously shown to contain the catalytic site of the glucose-6-phosphatase enzyme [6] in rat, rabbit, sheep, mouse and guinea pig hepatic microsomes [6]. Secondly, the 36.5 kDa labelled polypeptide doublet was specifically immunostained by antiserum previously shown to be monospecific for the rat microsomal glucose-6-phosphatase enzyme [17], and able to quantitatively immunoprecipitate glucose-6-phosphatase activity from cholate-solubilized rat hepatic microsomes [6]. Thirdly, the identification of the immunoreactive polypeptide in human hepatic microsomes as the glucose-6-phosphatase enzyme was confirmed by the complete absence of a cross-reacting protein in human hepatic microsomes isolated from a child who had previously been shown to have a type 1a glycogen storage disease using conventional assay methods [14].

The identification of the human hepatic microsomal glucose-6-phosphatase enzyme using the 36.5 kDa polypeptide(s) and the availability of a monospecific polyclonal antiserum provide powerful new tools to approach the purification of human protein and the isolation of human DNA probes for diagnosis, prenatal diagnosis and carrier detection of type 1a glycogen storage diseases.

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