Identification and characterization of a hepatic microsomal glucose transport protein

T₃ of the glucose-6-phosphatase system?

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A 52 kDa polypeptide in rat liver microsomes was identified as a glucose-binding protein by its ability to weakly bind cytochalasin B and by its cross-reactivity to an antibody raised against the human erythrocyte glucose transport protein. The microsomal glucose binding polypeptide was purified by affinity chromatography and an antibody was raised against it. The inhibitory effect of this antibody on rat microsomal glucose-6-phosphatase activity and on glucose transport out of microsomal vesicles indicates that this protein is a microsomal glucose transport protein.

INTRODUCTION

Glucose is the primary energy source for virtually all mammalian cells, and glucose transport across the plasma membrane is mediated by one or more of a family of glucose-specific transport proteins (e.g. Wheeler & Hinkle, 1985; Simpson & Cushman, 1986; Thorens *et al.*, 1988, 1990; Bell *et al.*, 1990). Blood glucose concentrations are maintained primarily by the balance between uptake of glucose into peripheral tissues and storage by or release from the liver (Ferrannini *et al.*, 1985). The hepatic microsomal glucose-6-phosphatase enzyme (EC 3.1.3.39) catalyses the terminal step of both gluconeogenesis and glycogenolysis and is therefore a key step in the regulation of blood glucose concentrations by the liver (Ashmore & Weber, 1959; Nordlie, 1985). Glucose-6-phosphatase is a multi-component system, with the catalytic subunit of the enzyme located within the lumen of the endoplasmic reticulum (ER), and a calcium-binding regulatory protein predominantly located on the cytosolic side of the ER. Three transport proteins are also required. T₁ mediates the entry of the substrate glucose 6-phosphate into the lumen of the ER, and T₂ is an antiport protein allowing the entry of an alternative substrate (pyrophosphate) and the exit of the product (phosphate) from the lumen of the ER (see Burchell, 1990, for a recent review). The route by which glucose, released by the action of glucose-6-phosphatase, leaves the lumen of the ER is an unresolved problem, but the protein involved has been designated 'T₃'. Genetic deficiencies of the enzyme, the regulatory protein, T₁ and T₂ have been described (see Burchell, 1990, for a recent review). In addition, the enzyme, the regulatory protein and T₂ have all been purified (see Burchell, 1990, for a recent review). In contrast, no deficiencies of T₃ have been described and nothing at all is known about the protein or proteins that transport glucose across the ER membrane. The existence of T₃ has become somewhat controversial, e.g. it has been suggested that glucose crosses the ER membrane by simple diffusion (Arion *et al.*, 1980), whereas kinetic data have indicated that T₃ does exist (Nordlie *et al.*, 1986). We have proposed that glucose transport across the ER membrane also requires the presence of a glucose transport protein or proteins (Burchell & Burchell, 1989). The aim of this study was therefore to determine whether or not an ER glucose transport protein(s) exists.

In this paper, we have used a number of techniques to identify two possible hepatic microsomal glucose transport proteins. One

of the proteins has now been purified to homogeneity, and an antibody raised against this protein was used to establish its role in the microsomal glucose-6-phosphatase system.

MATERIALS AND METHODS

Materials

Glucose 6-phosphate (monosodium salt), mannose 6-phosphate (monosodium salt), cytochalasin B, 2,5-diphenyloxazole, hydroxysuccinimidyl-4-azidobenzoate and prestained molecular mass markers were obtained from BDH, Poole, Dorset, U.K. [³H]Cytochalasin B, [U-¹⁴C]glucose, biotinylated donkey anti-sheep antibody, streptavidin-linked peroxidase complex and Hyperfilm-MP were purchased from Amersham International, Amersham, Bucks., U.K. Antiserum to the human erythrocyte glucose transport protein was a gift from Dr. S. A. Baldwin, Royal Free Hospital School of Medicine, London, U.K.

Empigen BB was kindly given by Albright and Wilson, Whitehaven, Cumbria, U.K. Nitrocellulose was obtained from Schleicher and Schuell, Dassel, Germany. Collagenase was from Boehringer Mannheim, Mannheim, Germany, and CNBr-activated Sepharose 4B was from Pharmacia, Uppsala, Sweden. All other chemicals were of the highest grade available.

Preparation of microsomal fractions

Rat and human liver microsomes were prepared as previously described (Burchell *et al.*, 1988), except that the microsomal pellet was resuspended to 20 mg/ml in 0.25 M-sucrose/25 mM-Tris acetate/20 mM-NaF, pH 7.4 (buffer A). The microsomes were then passed down a Sepharose 4B–concanavalin A affinity column as described by Semmi *et al.* (1982) to remove contamination with plasma membranes and disrupted microsomes.

Purification of the microsomal glucose transporter

Intact microsomal protein (80 mg) was carefully layered on to a 12–62% (w/v) linear 65 ml sucrose gradient in the presence of 25 mM-Tris acetate, 20 mM-NaF and 0.2% deoxycholic acid, pH 7.4, as previously described (Burchell & Cain, 1985). The gradient was centrifuged at 105 000 g for 18 h before the gradient was fractionated into 1 ml aliquots. Fractions containing glucose-6-phosphatase activity were pooled. A sample of the pooled fractions (2–3 ml) was passed down a Sephadex G-25M column which had previously been equilibrated with buffer A. Fractions

Abbreviations used: ER, endoplasmic reticulum; PBS, phosphate-buffered saline.

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containing protein were dialysed against phosphate-buffered saline (PBS, pH 8.0; Waddell & Burchell, 1988) overnight. Partially purified protein was loaded on to an anti-(human erythrocyte glucose transporter) antibody-Sepharose 4B affinity column and left for 1 h at room temperature. Non-specific proteins were removed by washing with PBS (pH 8.0). Affinity-purified protein was eluted by 3 ml of 0.1 M-glycine, pH 3.0. Protein content was estimated by the methods of Peterson (1977).

Glucose-6-phosphatase assays

Glucose-6-phosphatase and mannose-6-phosphatase activities were assayed and calculated as in Burchell *et al.* (1988), and are expressed as μmol of P_i released/min per mg of microsomal protein. Glucose 6-phosphate uptake into microsomes was measured as in Waddell & Burchell (1987) and Waddell *et al.* (1989).

Antiserum to the 52 kDa polypeptide

An antiserum to the purified 52 kDa polypeptide was raised in an adult Cheviot sheep as previously described (Waddell & Burchell, 1988). IgG was further purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Fahey & Terry, 1978).

Immunoblot analysis

SDS/PAGE was carried out in 7–16% (w/v) polyacrylamide gels as described by Laemmli (1970). Proteins separated on SDS/polyacrylamide gels were electrophoretically transferred to nitrocellulose, as described by Towbin *et al.* (1979). The Western blot was immunostained with a sheep anti-(rat microsomal glucose binding protein) IgG or an anti-(human erythrocyte glucose transport protein) IgG. Immunoreactive polypeptides were detected by using a biotin-streptavidin-peroxidase-linked detection system with 4-chloro-1-naphthol as the substrate (Mandrell & Zollinger, 1984; Domin *et al.*, 1984).

Determination of glucose transporter number

The number of D-glucose-inhibitable cytochalasin B-binding sites in the microsomal fractions was determined as previously described (Cushman & Wardzala, 1980).

Photochemical cross-linking of membranes

Microsomal fractions, either intact or fully disrupted by deoxycholate as described by Burchell & Cain (1985), were resuspended in 30 mM-Tris/HCl (pH 7.4) to a final concentration of 1–5 mg of membrane protein/ml. The membranes were incubated at 4 °C for 10 min with $[\text{H}]\text{cytochalasin B}$ at a final concentration of 10 μM . The membranes were then photochemically cross-linked with hydroxysuccinimide-4-azido-benzoate (400 μM) by exposure to u.v. light (254 nm) for 2 min as previously described (Horuk *et al.*, 1983). Control experiments were as above, except that 500 mM-D-glucose was incubated with the membranes for 30 min before the addition of cytochalasin B.

Membranes covalently cross-linked to $[\text{H}]\text{cytochalasin B}$ were immediately solubilized by boiling for 4 min in Laemmli (1970) sample buffer and were then subjected to SDS/PAGE on a 7–16% gel. Radioactive bands were detected either by fluorography using 2,5-diphenyloxazole (PPO) (Bonner & Laskey, 1974) or by scintillation counting of gel slices.

RESULTS AND DISCUSSION

Two approaches were attempted to try and identify microsomal glucose transport proteins. First, immunoblot analysis of microsomes from which plasma membranes had been removed were carried out using antibody raised against the human erythrocyte

Fig. 1. Immunoblot analysis using the anti-(human erythrocyte glucose transporter) antibody

Lanes: 1, human hepatic microsomes (30 μg); 2, liver microsomes (25 μg) prepared from starved rats.

glucose transport protein (Baldwin *et al.*, 1982). Fig. 1 shows that two bands which are present in both rat and human microsomes cross-react weakly with the antibody. The molecular masses of the bands were approx. 52 kDa and 24 kDa.

Cytochalasin B is a potent reversible inhibitor of plasma membrane glucose transport proteins, and binding of cytochalasin B to these proteins is blocked by D-glucose. Cytochalasin B has been widely used as a probe to identify and characterize glucose transport proteins in plasma membranes (e.g. Lin & Spudich, 1974; Jung & Rampal, 1977; Baldwin *et al.*, 1979; Shanahan, 1982, 1983; Kasanicki *et al.*, 1987; Wang, 1987; Chen & Lo, 1988; Oka *et al.*, 1988). The amount of cytochalasin B that inhibits erythrocyte plasma membrane glucose transport half-maximally is 0.07–0.04 μM (Jung & Rampal, 1977; Axelrod & Pilch, 1983), whereas the K_i of 1.9 μM for inhibition of hepatic plasma membrane glucose uptake is an order of magnitude higher (Axelrod & Pilch, 1983). It had previously been reported that cytochalasin B does not inhibit D-glucose transport across the ER membrane (Arion *et al.*, 1980), although no details of the concentrations of cytochalasin B used were given, as this was a reference to other work which has not yet been published. We therefore carried out studies of cytochalasin B inhibition of glucose-6-phosphatase activity in intact microsomes. We found that cytochalasin B was a very poor inhibitor of glucose-6-phosphatase activity. However, very high levels (20 μM) of cytochalasin B (an order of magnitude higher than are required for the plasma membrane glucose transport protein; see above) did have a small inhibitory effect (mean $26 \pm 2\%$) on glucose-6-phosphatase activity in intact microsomes, suggesting that cytochalasin B might bind weakly to T_3 , the proposed microsomal glucose transport protein of the glucose-6-phosphatase system. T_3 is not normally the rate-limiting step of the glucose-6-phosphatase system in intact microsomes (Arion *et al.*, 1980). T_3 would therefore have to be highly inhibited to produce a relatively small inhibition of glucose-6-phosphatase activity in intact microsomal vesicles. It is therefore not possible to directly calculate the K_i of cytochalasin B inhibition of T_3 .

The number of glucose transporters in the hepatic subcellular membrane fractions was assessed by the D-glucose-inhibitable $[\text{H}]\text{cytochalasin B}$ binding assay. The number of glucose transporters/mg of membrane protein in the microsomal fraction was 6 ± 3 pmol/mg, with a K_d of 931 μM . The number of glucose transporters/mg of plasma membrane protein was approx. 8-fold higher (46 ± 2 pmol/mg), with a K_d of 701 μM . These values are in good agreement with previously reported values (Ciaraldi *et al.*,

1986). The values obtained suggest that there is only a low number of glucose transport protein molecules in the microsomal membrane; however, we cannot rule out the possibility that all of the glucose transport molecules in the microsomal membrane may not be available for cytochalasin B binding. That this is a possibility has been suggested by two studies of the human erythrocyte transport protein. The degree of glycation of the erythrocyte transport protein has been shown to alter the ability of the protein to bind cytochalasin B (Bilan & Klip, 1990). Secondly, the human erythrocyte glucose transport protein has been shown to bind to glyceraldehyde-3-phosphate dehydrogenase (Lachaal *et al.*, 1990). The fact that glucose transport proteins can bind to other proteins increases the possibility that T_3 may be able to bind directly to other components of the glucose-6-phosphatase system. The way we purified T_3 from a partially purified preparation of the glucose-6-phosphatase enzyme (see below) also indicates that this may be the case.

The second approach that was used to identify microsomal glucose transport protein(s) was to attempt to label such proteins with [3 H]cytochalasin B in the presence and absence of glucose, as described by Horuk *et al.* (1983) for the labelling of plasma membrane glucose transport proteins. Fig. 2(a) shows that three peaks of radioactivity were observed in gel slices after SDS/PAGE. The first peak, which is of very high molecular mass (> 100 kDa), is also seen in control microsomes and is presumably non-specific binding. The other two peaks, at 48–52 kDa and 22–24 kDa, were D-glucose-inhibitable and correspond well with those observed in Fig. 1.

The labelling with cytochalasin B was very weak, as was expected from the studies of cytochalasin B inhibition of microsomal glucose-6-phosphatase activity. Fluorography of SDS gels revealed two bands of approx. 52 and 24 kDa, but the amount of labelling with [3 H]cytochalasin B was so low that only faint bands were visible on the films (Fig. 2).

The fact that two completely independent techniques both identified the same two bands of approx. 52 and 24 kDa as probable glucose binding or glucose transport proteins led us to attempt to purify the two proteins.

Fig. 2. Photoaffinity labelling of microsomal protein with cytochalasin B

(a) Photoaffinity cross-linking was carried out as described (Horuk *et al.*, 1983). Control samples (◆), but not test samples (□), were exposed to 10 mM-D-glucose before addition of the cross-linking agent. Samples were then electrophoresed on a 7–16% (w/v) polyacrylamide gel and each lane was sliced into 0.5 cm segments or placed into acetic acid for fluorography. After storage in tissue solubilizer overnight, radioactivity was estimated by liquid scintillation counting. Each point represents the mean of three lanes. Molecular mass values are indicated above the Figure. (b) Fluorograph of a test lane. After fixing in glacial acetic acid, gel slices were incubated in 40% 2,5-diphenyloxazole (PPO)/acetic acid for 2 h, and washed in acetic acid and water before being dried down and autoradiographed against Hyperfilm-MP for 91 days.

Fig. 3. Immunoblot analysis of rat and human microsomes using anti-(52 kDa protein) antibody

Microsomes were run on a 7–16% (w/v) polyacrylamide gel before transfer to nitrocellulose and immunoblotting with anti-(52 kDa protein) antibody. Lane 1, liver microsomes prepared from starved rats (10 μg); lane 2, human liver microsomes (15 μg).

In order to confirm that the protein(s) purified had a role in the glucose-6-phosphatase system, we partially purified the glucose-6-phosphatase enzyme using sucrose gradients, as described in the Materials and methods section. It seemed possible that T_3 , the proposed glucose transport protein, might co-purify with the catalytic subunit activity of glucose-6-phosphatase during this procedure, as the regulatory stabilizing protein, the catalytic subunit protein and the T_2 protein are all present in the pooled fractions. The pooled fractions were then purified further using an anti-(human erythrocyte glucose transport protein) IgG affinity column as described in the Materials and methods section. Only one protein, as judged by protein staining of SDS/polyacrylamide gels, bound to the column and was eluted with 0.1 M-glycine, pH 3.0. The purified protein, which had a molecular mass of 52 kDa, was used to raise an antiserum in sheep. The antiserum was monospecific for a single 52 kDa polypeptide in rat and human microsomes as judged by immunoblot experiments (Fig. 3). The molecular mass of the band was found to be 52 kDa, as judged by SDS/polyacrylamide gel electrophoresis on 10% (w/v) polyacrylamide gels. On gradient gels, e.g. Fig. 3, which is a 7–16% polyacrylamide gel, the apparent molecular mass of the band was somewhat variable. Other facilitated glucose transporters are well known to be glycosylated (e.g. see Bilan & Klip, 1990). Assuming that the microsomal glucose transporter is also a glycoprotein, the carbohydrate content of the protein may contribute to the fact that its apparent molecular mass varies slightly depending on the precise percentage of acrylamide present in polyacrylamide gels.

In order to determine whether the 52 kDa polypeptide is a protein involved in the glucose-6-phosphatase system, the antiserum raised against the 52 kDa protein was incubated with intact rat liver microsomes. The antiserum inhibited the V_{max} of glucose-6-phosphatase activity by up to 36% (see Table 1), but had no significant effect on the K_m of glucose-6-phosphatase in intact rat liver microsomes. The K_m remained constant at 3.2 ± 0.1 mM ($n = 26$) in the presence of both pre-immune and test antiserum (see Table 1). Addition of control pre-immune antiserum from the same sheep caused a small increase in glucose-6-phosphatase activity in intact microsomes. No inhibition was seen when the antiserum was incubated with fully disrupted rat liver microsomes (results not shown). The inhibition of glucose-

Table 1. Effect of sheep anti-(52 kDa microsomal protein) antibody on glucose-6-phosphatase activity in intact liver microsomes prepared from starved rats

Glucose-6-phosphatase activity is expressed as a percentage of the control (V_{max}) glucose-6-phosphatase activity, which was 0.18 $\mu\text{mol/min}$ per mg of protein. The K_m (3.2 ± 0.1 mM) did not change significantly in the presence of antiserum. Values are the means $\pm$ S.E.M. of at least three different sets of determinations, each of which was carried out in duplicate.

Serum additions ($\mu\text{g/ml}$)	Glucose-6-phosphatase activity (%)	
	Pre-immune serum	Test serum
0	100	100
0.1	111 $\pm$ 5	91 $\pm$ 1
0.12	116 $\pm$ 12	64 $\pm$ 3
0.14	119	75 $\pm$ 6
0.16	111 $\pm$ 5	78 $\pm$ 6

Fig. 4. Effect of anti-(52 kDa protein) antibody on rat hepatic microsomal [^{14}C]glucose-6-phosphate (G-6-P) uptake

The graph shows the uptake of [^{14}C]G-6-P into starved rat liver microsomes. Transport assays were carried out as described in Waddell *et al.* (1989). Incubations were carried out in 40 mM-cacodylate buffer/1 mM-G-6-P/0.5 μCi of [^{14}C]G-6-P/ml. Two controls (■, ●) contained 40 μg of preimmune serum/ml, while the test (○) contained 40 μg of anti-(52 kDa protein) antibody/ml. After 3 min (arrow), a chase of 25 mM unlabelled G-6-P was added to the test (○) and to one control (●).

6-phosphatase activity by the antiserum indicates that the 52 kDa polypeptide has a role in glucose-6-phosphatase activity. Inhibition of T_3 would lead to an increase in the glucose concentration inside the lumen of the ER. Glucose is a non-competitive inhibitor of the glucose-6-phosphatase enzyme (e.g. see Arion *et al.*, 1980), so the increased luminal glucose would inhibit glucose-6-phosphatase in intact microsomes, resulting in a decreased V_{max} , as seen in Table 1. The small V_{max} inhibition caused by the antiserum is therefore compatible with the 52 kDa glucose binding and/or transport protein being T_3 . Direct transport assays with glucose-6-phosphate as substrate were carried out as previously described (Waddell & Burchell, 1987; Waddell *et al.*, 1989). Fig. 4 shows that the antiserum raised against the 52 kDa protein decreases the rate of [^{14}C]glucose release from starved rat liver microsomes which have been incubated with [^{14}C]glucose 6-phosphate, followed by the addition of 25 mM non-radioactive glucose 6-phosphate.

In conclusion, the 52 kDa protein that we have purified from rat liver microsomes is a glucose transport protein that plays a

role in the activity of glucose-6-phosphatase in intact rat liver microsomes. It is therefore most probably T_3 , the glucose transport protein of the glucose-6-phosphatase system. The very weak binding of cytochalasin B to the 52 kDa protein compared with the binding of other plasma membrane glucose transport proteins suggests that the microsomal 52 kDa protein (T_3) is a novel glucose transport protein. It will be necessary to isolate a full-length rat liver cDNA coding for the 52 kDa protein to establish its similarity to the plasma membrane glucose transport proteins (GLUTs 1–5) (Bell *et al.*, 1990) and for use in studies of type 1 glycogen storage disease patients. Further studies must also be undertaken to purify the 24 kDa microsomal protein, which binds cytochalasin B and cross-reacts with anti-(erythrocyte glucose transport protein) antibody but not with the antibody raised against the 52 kDa microsomal protein, to determine whether it also is needed for normal glucose-6-phosphatase activity in intact microsomes.

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