Identification of protein components of the microsomal glucose 6-phosphate transporter by photoaffinity labelling

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The glucose-6-phosphatase system catalyses the terminal step of hepatic glucose production from both gluconeogenesis and glycogenolysis and is thus a key regulatory factor of blood glucose homeostasis. To identify the glucose 6-phosphate transporter T1, we have performed photoaffinity labelling of human and rat liver microsomes by using the specific photoreactive glucose-6-phosphate translocase inhibitors S 0957 and S 1743. Membrane proteins of molecular mass 70, 55, 33 and 31 kDa were labelled in human microsomes by [³H]S 0957, whereas in rat liver microsomes bands at 95, 70, 57, 54, 50, 41, 33 and 31 kDa were detectable. The photoprobe [³H]S 1743 led to the predominant labelling of a 57 kDa and a 50 kDa protein in the rat. Stripping of microsomes with 0.3% CHAPS retains the specific binding of T1 inhibitors; photoaffinity labelling of such CHAPS-

treated microsomes resulted in the labelling of membrane proteins of molecular mass 55, 33 and 31 kDa in human liver and 50, 33 and 31 kDa in rat liver. Photoaffinity labelling of human liver tissue samples from a healthy individual and from liver samples of patients with a diagnosed glycogen-storage disease type 1b (GSD type 1b; von Gierke's disease) revealed the absence of the 55 kDa protein from one of the patients with GSD type 1. These findings support the identity of the glucose 6-phosphate transporter T1, with endoplasmic reticulum protein of molecular mass 50 kDa in rat liver and 55 kDa in human liver.

Key words: chlorogenic acid, endoplasmic reticulum, glucose-6-phosphatase, hepatic glucose output, photoaffinity labels.

INTRODUCTION

In mammals the liver is the organ responsible for the homeostatic regulation of blood glucose levels [1–3]. Glucose-6-phosphatase (EC 3.1.3.9) catalyses the terminal step of hepatic glucose production by the hydrolysis of glucose 6-phosphate deriving from either gluconeogenesis or glycogenolysis in the endoplasmic reticulum of hepatocytes [1]. Two alternative models have been proposed for the glucose-6-phosphatase system. The multicomponent substrate transport model comprises a protein complex involving a phosphohydrolase with its active site oriented towards the lumen of the endoplasmic reticulum, and transporter proteins for glucose 6-phosphate (T1) and phosphate (T2) [4–6]. The alternative model, the 'combined conformational flexibility-substrate-transport model', hypothesizes a single, membrane-spanning, protein with its active site directly accessible to glucose 6-phosphate; the enzyme's catalytic activity is conformationally constrained by the intact membrane, and translocation of glucose 6-phosphate into the lumen of the endoplasmic reticulum would not be required [7–9]. Excessive endogenous hepatic glucose production contributes significantly to fasting hyperglycaemia in type 2 diabetic patients [10,11]. Inhibition of the glucose-6-phosphatase at the terminus of the major glucogenic pathways therefore provides an attractive approach to therapeutic intervention. In terms of the substrate-transport model, glucose 6-phosphate transport across the membrane of the endoplasmic reticulum shows a high specificity for glucose 6-phosphate because it does not transport its 2-epimer mannose 6-phosphate. The glucose 6-phosphate transporter T1 is therefore an ideal target for pharmacological intervention. We have previously identified a new class of highly specific inhibitors of microsomal glucose 6-phosphate transport.

These derivatives of chlorogenic acid are able to decrease hepatic glucose production and lower blood glucose levels in normal and diabetic animal models [12–18].

Glycogen-storage disease type 1 (GSD 1; von Gierke's disease) is an inborn error of metabolism caused by the deficiency of glucose-6-phosphatase and is characterized by severe hypoglycaemia, hepatomegaly, growth retardation, lactic acidemia, hyperlipidaemia and hyperuricaemia [2,19]. On the basis of biochemical and clinical heterogeneity, four subtypes of GSD 1 are currently distinguished [19]. GSD type 1a could be characterized as caused by mutations in the glucose-6-phosphatase gene [20], whereas in GSD type 1b patients there is evidence for defects in the glucose 6-phosphate transport [5,21]. A candidate gene for the human glucose-6-phosphate translocase has recently been described [22] but a functional relationship to glucose 6-phosphate transport was not established.

Chlorogenic acid derivatives are highly specific competitive inhibitors of glucose-6-phosphatase activity in intact rat and human microsomes, leaving the phosphohydrolase activity of detergent disrupted microsomes unaffected [14,15]. Recently, with the use of radiolabelled analogues of chlorogenic acid a high-affinity binding site could be identified in rat liver microsomes fitting all the required criteria for the postulated glucose-6-phosphate translocase T1, namely glucose 6-phosphate competitively blocked binding, whereas binding was unaffected by mannose 6-phosphate. In detergent-disrupted microsomes, glucose 6-phosphate is hydrolysed at a site that is unaffected by chlorogenic acid analogues. Most significantly, permeabilization of the membranes with detergent activated glucose-6-phosphatase and broadened its substrate specificity but did not alter binding of the analogue to T1; neither did it affect the ability of glucose 6-phosphate to block binding [15]. In the present study,

Abbreviations used: DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; GSD type 1, glycogen-storage disease type 1 (von Gierke's disease).

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the tight binding between the more potent chlorogenic acid derivatives with integral membrane proteins of liver microsomes was further exploited to investigate the molecular structure of the glucose-6-phosphatase system directly. In the study described here, we used photolabile chlorogenic acid analogues to identify intrinsic membrane proteins that contain tight binding sites for this class of functional glucose-6-phosphate translocase inhibitors in rat and human liver microsomes. This allowed the identification, for the first time, of functional protein components of the postulated glucose-6-phosphate translocase T1 in human and rat liver. Part of this work has been presented as abstracts [23,24].

EXPERIMENTAL

Materials

Marker proteins for the determination of molecular masses were from Sigma (Munich, Germany). Triton X-100®, CHAPS, Serva Blue R-250 and Western-light chemiluminescence detection kit and all other materials for electrophoresis were from Serva (Heidelberg, Germany). Antibodies raised against glucose-6-phosphatase were kindly provided by Dr. Rebecca Taub (University of Pennsylvania, Philadelphia, PA, U.S.A.) [25]. Polyclonal anti-(glucose-6-phosphate translocase) antibodies were generated in rabbit by Eurogentec (Liège, Belgium) against a synthesized peptide (residues 203–216 of the rat homologue of the described human gene) [22], and P. Habermann, unpublished work). The sequence of the peptide was confirmed. Enhanced anti-(glucose-6-phosphate translocase) activity was confirmed in ELISA assays with immobilized glucose-6-phosphate translocase peptide. Nitrocellulose membranes (0.2 µM trans-blot transfer medium) for immunoblotting and the protein determination kit (Bradford method [26]) were from Bio-Rad (Munich, Germany). Scintillator Quickzint 501 and tissue solubilizer Biolute S were purchased from Zinsser Analytic GmbH (Frankfurt, Germany).

Photoaffinity probes

Photoaffinity probes S 0957 and S 1743 (Figure 1) were synthesized in accordance with the procedures described [12,13]. [³H]S 0957 was synthesized by the addition of 20 µl (100 µCi) of 4-azido[3,5-³H]benzoyl-*N*-hydroxysuccinimide ester (NEN-DuPont; specific radioactivity 50 Ci/mmol) in dioxane to 1 mg of (1*S*,3*R*,4*R*,5*R*)-3-imidazo[4,5-*b*]pyridin-1-yl-3-phenylacrylic acid 5-(4-aminobutane-1-sulphonylamino-carbonyl)-5-[2-(4-chlorophenyl)-cyclopropylmethoxy]-2,3-(dioxospirocyclohexyl)-cyclohexyl ester in 20 µl of dioxane plus 1.65 µl of triethylamine. After 12 h at 20 °C the reaction mixture was applied to a high performance (HP)TLC plate (20 cm × 5 cm) and the chromatogram was developed in toluene/methanol/acetic acid (10:2:1, by vol.) as solvent. After detection of radioactivity with a radiochromatogram scanner (Berthold, Calw, Germany) the radioactive band was scraped off and the compound was eluted with 1500 µl of ethanol (yield at least 70 %) and stored at 4 °C (approx. 0.5 µCi/10 µl). For the preparation of [³H]S 0957 the appropriate amount was removed from stock solution and 5 µl of 0.1 M HCl was added to remove the cyclohexylidene protecting group. Deprotection was monitored by HPTLC in the above solvent. After completion of deprotection (24–40 h) the solvent was evaporated to dryness and appropriate buffer was added to achieve the desired concentration of [³H]S 0957. This solution was used for the photoaffinity labelling experiments immediately after preparation. The photoaffinity probe [³H]S 1743 (specific radioactivity 5 Ci/mmol) was synthesized by acetylation of [1*S*,3*R*,4*R*,5*R*]-3-imidazo[4,5-*b*]pyridin-1-yl-3-phenylacrylic

acid-5-(4-aminobutane-1-sulphonylamino-carbonyl)-5-[2-(4-chlorophenyl)-cyclopropylmethoxy]-2,3-(dioxospirocyclohexyl)-cyclohexyl ester with [³H]acetic anhydride. Deprotection to the photoaffinity probe [³H]S 1743 was performed as with [³H]S 0957.

Human tissues

Fragments of control liver from a 35-year-old female organ donor were provided by the department of Pharmacokinetics and Metabolism of Hoechst Marion Roussel Deutschland GmbH (Frankfurt am Main, Germany). The GSD type 1b liver samples were generously provided by Professor Van Schaftingen. Surgical biopsy specimens were obtained from two female patients at the age of 10 years (patient 1; patient V. K. in [22]) and 22 years (patient 2; patient G. L. in [22]) and were kept at –80 °C before use. The patients showed typical clinical and laboratory symptoms of GSD 1b. According to Professor Van Schaftingen, the biochemical diagnosis of GSD type 1b was based on the finding of normal total glucose-6-phosphatase activity and the existence of a significant abnormal latency (where latency is defined as the increased enzymic activity observed on disruption of the membrane by detergents) [5]. Glucose-6-phosphatase activity that could be released from biopsy samples was normal in detergent-treated extracts, but was lower in homogenates from fresh liver. Furthermore, the rate of [U-¹⁴C]glucose 6-phosphate uptake was approx. 10 % of control values [22].

Preparation of rat liver microsomes

Hepatic microsomes were prepared as described previously and stored frozen at –70 °C at a protein concentration of 15–20 mg/ml [14]. Rat liver microsomes were prepared from rats fasted for 24 h. The intactness of the preparations, measured by the latency of mannose-6-phosphatase activity [27], was usually above 97 %. Partly stripped ('disrupted') microsomes were prepared with the use of published procedures [14] by exposing thawed microsomes (2 mg/ml protein) for 20 min at 4 °C to 0.3 % (w/v) CHAPS. Under these conditions, latent activity was released maximally, whereas the glucose-6-phosphatase catalytic unit was not affected (results not shown). We evaluated the specific inhibitory potency of chlorogenic acid analogues on the glucose-6-phosphate translocase by assessing their effect on the phosphohydrolase activity of 'untreated' and disrupted microsomes. (The term 'untreated' is used to designate microsomal vesicles that are prepared and used without further treatment.) In untreated microsomes from fasted rats, T1 translocase imposes significant rate limitations on the steady-state hydrolysis of glucose 6-phosphate, whereas under these conditions other translocase components do not play a significant role. The hydrolysis of PP_i by untreated microsomes provided an assay for T2, the phosphate translocase [4]. The hydrolysis of glucose 6-phosphate, mannose 6-phosphate and sodium pyrophosphate was assayed by the quantification of P_i by a colorimetric assay described previously [28] scaled down to accommodate assay volumes of 300 µl. Data points in the figures are the means of two assay values. IC₅₀ values were obtained by non-linear least-squares analysis of plotted data with the use of Microcal Origin 3.5 (Microcal Software, Northampton, MA, U.S.A.). IC₅₀ values are the means of at least three independent assays.

Photoaffinity labelling

Photoaffinity labelling was performed in a Rayonet RPR-100 photochemical reactor (The Southern Ultraviolet Company,

Hamden, CT, U.S.A.) equipped with RPR 3000 Å or RPR 2537 Å lamps. For photoaffinity labelling, human or rat liver microsomes or detergent-treated microsomes (100–300 µg of microsomal protein) were incubated at 20 °C for 5 min in the dark with the appropriate concentration of [³H]S 0957 in a total volume of 200 µl of 50 mM Hepes/HCl buffer (pH 7.0)/250 mM sucrose followed by irradiation (20 s at 254 nm or 1 min at 300 nm). For photoaffinity labelling of human liver tissue, 40 mg of frozen biopsy samples was homogenized in the above buffer followed by centrifugation at 100000 g for 30 min. The resulting pellet was resuspended in buffer and used immediately for experiments. After photoaffinity labelling, microsomes or particulate material were washed twice with the above buffer, proteins were precipitated [29] and analysed by SDS/PAGE [30,31]. Phase separation experiments to discriminate between integral and peripheral membrane proteins by alkaline extraction or treatment with non-ionic detergents were performed as described previously [30]. Radioactively labelled polypeptides were detected either by liquid-scintillation counting after the gels had been sliced into 2 mm pieces with subsequent hydrolysis of polypeptides with tissue solubilizer Biolute S or by fluorography as described [31].

Immunoblotting

Proteins were transferred to nitrocellulose membranes (0.2 µm trans-blot transfer medium from Bio-Rad) from SDS/PAGE gels in a trans-blot cell (Bio-Rad) by using 25 mM Tris/HCl/192 mM glycine/33% (v/v) methanol (pH 8.3). A 1:1000 dilution of anti-(glucose-6-phosphate translocase) serum followed by a 1:10000 dilution of anti-rabbit serum and the Western-light chemiluminescence detection system from Serva, in accordance with the protocol of the manufacturer, was used for immunodetection.

RESULTS

Interaction of the photoreactive chlorogenic acid derivatives with microsomal glucose-6-phosphatase activity

Workers in our laboratories have identified derivatives of chlorogenic acid that are potent inhibitors of glucose 6-phosphate hydrolysis by intact microsomes with IC₅₀ values in the sub-micromolar range [13–15]. The chlorogenic acid analogues had no effect on the glucose-6-phosphatase activity by detergent-disrupted microsomes; neither did they inhibit the pyrophosphatase activity by untreated microsomes. In terms of the substrate-transport model, the compounds are site-specific inhibitors of T1, the glucose 6-phosphate transporter of liver and renal microsomes [14–16] without an effect on the glucose-6-phosphatase enzyme or on T2, the postulated P_i/PP_i transporter. Detailed kinetic analysis revealed that chlorogenic acid or its high-affinity analogues such as S 3483 or S 5627 are reversible linear competitive inhibitors. Concentration-dependent inhibition of glucose 6-phosphate translocation by chlorogenic acid analogues has also been demonstrated in microsomal vesicles by using a light scattering procedure ([32], and H.-J. Burger, unpublished work). In addition to their therapeutic potential, these tightly binding inhibitors of T1 are useful new tools for investigating the molecular structure of the glucose 6-phosphate system. Their high affinity should allow the identification of the protein components of the glucose-6-phosphatase system by affinity labelling or photoaffinity labelling techniques. We therefore synthesized S 0957 and S 1743, two photoreactive analogues of chlorogenic acid, with high specific radioactivities (Figure 1).

Figure 1 Structures of the photoreactive chlorogenic acid derivatives [³H]S 0957 and [³H]S 1743

Figure 2 Interaction of photoreactive chlorogenic acid derivatives with intact and Triton-disrupted microsomes

Glucose-6-phosphatase and pyrophosphatase were assayed by quantifying the formation of P_i [18] in 'untreated' or Triton X-100-disrupted rat liver microsomes (5 µg of protein; 50 µg/ml) incubated in 50 mM Hepes/HCl buffer (pH 7.0)/250 mM sucrose (10 min at 30 °C) in the dark with 1 mM glucose 6-phosphate (●, 'untreated'; ○, 'disrupted') or 1 mM PP_i (▲, 'untreated') in the presence of the indicated concentrations of S 0957. Sigmoid lines were fitted by unweighted non-linear regression. Representative control values in the absence of test compound were 140, 522 and 120 nmol/min per mg of protein for glucose-6-phosphatase (untreated), glucose-6-phosphatase (disrupted) and pyrophosphatase (untreated) respectively.

Figure 3 Photoaffinity labelling of liver microsomes with [^3H]S 0957

Left panel: rat liver microsomes (300 μg of protein; 0.75 mg/ml) were incubated for 5 min at 20 $^{\circ}\text{C}$ in the dark with 6.25 nM (0.1 μCi), 15.54 nM (0.25 μCi), 31.75 nM (0.5 μCi), 62.5 nM (1 μCi) or 125 nM (2 μCi) [^3H] S 0957 (as indicated) in 50 mM HEPES/HCl buffer (pH 7.0)/250 mM sucrose; they were then irradiated for 30 s at 300 nm. After the microsomes had been washed, 150 μg of protein was analysed by SDS/PAGE [12% (w/v) gel] followed by slicing of the gel lanes and detection of radioactivity by liquid-scintillation counting of hydrolysed proteins. Right panel: human liver microsomes (100 μg of protein) (lane A), human liver microsomal membranes after treatment with 0.3% CHAPS (lane B) or rat liver microsomes treated with 0.3% CHAPS (lane C) were irradiated for 20 s at 254 nm with 200 nM (1.5 μCi) [^3H]S 0957 followed by SDS/PAGE (100 μg of protein per lane) and the detection of radioactivity by fluorography. The positions of molecular mass markers are indicated (in kDa) at the left; the molecular masses of the bands are indicated at the right.

A prerequisite for photoaffinity labelling is that the respective photolabile analogues show as specific an interaction with its binding proteins as do the non-modified ligands. Figure 2 shows the concentration–response curves of S 0957 on the glucose-6-phosphatase activity of rat liver microsomes. S 0957 inhibited glucose-6-phosphatase activity in untreated rat liver microsomes with an IC_{50} of 160 ± 40 nM when assayed at 1 mM glucose 6-phosphate. S 0957 did not inhibit the hydrolysis of 1 mM PP_i in untreated microsomes, which excluded P_i/PP_i translocases as the site of inhibition. S 0957 also showed no effect on the phosphohydrolase activity when assayed at 1 mM glucose 6-phosphate in microsomes after complete disruption of the membranes with 0.1% Triton X-100 (Figure 2). Disruption of microsomes with other detergents such as 0.3% CHAPS had the same effects (results not shown). Under the same experimental conditions, compound S 1743 also inhibited only the microsomal glucose 6-phosphate transporter T1 with an IC_{50} of 86 nM in the assay of untreated microsomes with 1 mM glucose-6-phosphatase. These preliminary characterizations indicate that both of the photolabile inhibitors block glucose-6-phosphatase activity associating

with the glucose-6-phosphate binding site on T1, thus allowing the identification of its protein components.

Suitability of photoreactive chlorogenic acid derivatives for photoaffinity labelling

The photoreactive transport inhibitor [^3H]S 0957 carries an aromatic azidophenyl moiety that can be activated to a highly reactive aromatic nitrene on irradiation with UV at 254 or 300 nm with a half-life of 4–5 s. To determine the suitability of [^3H]S 0957 for covalent photo-cross-linking to the respective binding proteins, solutions of BSA were incubated with sub-micromolar concentrations of the photoprobes followed by irradiation at 254 or 300 nm. The proteins were subsequently separated by SDS/PAGE; the incorporation of the respective probe into albumin was determined by fluorography as well as by slicing of the gels and the quantification of protein-bound radioactivity by liquid-scintillation counting. After 1 min of photolysis, the cross-linking rate was high at 254 or 300 nm,

Figure 4 Photoaffinity labelling of rat liver microsomes with [^3H]S 1743

Intact rat liver microsomes (150 μg of protein, 0.75 $\mu\text{g}/\text{ml}$) were incubated for 5 min at 20 $^{\circ}\text{C}$ in the dark with 100 nM (1 μCi) [^3H]S 0957 or 1.33 μM (1 μCi) [^3H]S 1743. After irradiation for 20 s (S 0957) or 1 min (S 1743) at 254 nm as well as for 5 min at 300 nm, the microsomes were washed and membrane proteins were separated by SDS/PAGE [12% (w/v) gel]. Left panels: distribution of radioactivity by liquid-scintillation counting after the gel lanes had been cut into 2 mm slices and the proteins hydrolysed with Biolute S: top panel, after labelling with [^3H]S 0957 at 254 nm for 20 s; lower panel, after labelling with [^3H]S 1743 at 254 nm for 1 min. The molecular masses of the peaks (in kDa) are indicated. Right panel: detection of radioactivity by fluorography (15 weeks of exposure). Lane A, after labelling with [^3H]S 0957 at 254 nm for 20 s; lane B, after labelling with [^3H]S 1743 at 254 nm for 1 min; lane C, after labelling with [^3H]S 1743 at 300 nm for 5 min. The positions of molecular mass markers are indicated (in kDa) at the left; the molecular masses of the bands are indicated at the right.

whereas irradiation at 350 nm led to less than half of the incorporation rate, suggesting that [^3H]S 0957 should be suited to identification of the protein components of T1. In contrast with S 0957, the inhibitor S 1743 does not contain an aromatic azido function. Owing to the presence of the aromatic and heteroaromatic structures with delocalized π -electron systems, S 1743 seems suited to act as a direct photoaffinity probe. To investigate this possibility, we performed cross-linking studies with albumin solutions. On irradiation at 254 or 300 nm, covalent labelling of the binding protein albumin was achieved, with the highest incorporation at 254 nm. To minimize the photochemical damage of microsomal proteins, an irradiation time of 20–30 s at 254 or 300 nm for [^3H]S 0957 and 1 min at 254 nm was chosen for photoaffinity labelling experiments of microsomes with [^3H]S 1743.

Photoaffinity labelling of rat and human liver microsomes

To identify the putative protein components of the glucose 6-phosphate transporter T1, microsomal protein fractions isolated from rat liver and human liver were subjected to photoaffinity labelling with [^3H]S 0957 followed by SDS/PAGE and scrutiny of the gels in 2 mm pieces, with determination of radioactivity by liquid-scintillation counting after hydrolysis of proteins with alkaline tissue solubilizer. Photoaffinity labelling of rat liver microsomes with increasing concentrations of [^3H]S 0957 in the concentration range 6.25–125 nM led to the labelling of three polypeptide bands of molecular mass 32, 54 and 70 kDa; the 32 kDa band was labelled even at the lowest concentration (Figure 3, left panels). None of these radiolabelled proteins is identical with the glucose-6-phosphatase enzyme as was demonstrated by immunoblotting with anti-(glucose-6-phosphatase)

antibodies (results not shown). Radiolabelled proteins migrating less than 2 mm apart on SDS gels could escape identification by detection of radioactivity by slicing of the gels [30]. We therefore used fluorography as a second method to identify radioactively labelled proteins after gel electrophoresis. Fluorographic detection of microsomes photolabelled with [^3H]S 0957 revealed that the bands identified by slicing of the gels were resolved into several additional bands on the fluorograms: the 32 kDa band consisted of two polypeptides of molecular mass 31 and 33 kDa, whereas the 54 kDa band contained several polypeptides of molecular mass 57, 54 and 50 kDa; additional faint bands at molecular mass 41 and 95 kDa were also detected in some experiments (see Figures 4–7). With microsomes prepared from healthy human liver a very similar labelling pattern occurred, suggesting great similarity between the respective binding proteins of human and rat. Fluorographic detection of microsomes from human liver labelled with [^3H]S 0957 showed prominent labelling of a 55 kDa polypeptide as well as of 33 and 31 kDa proteins, with an additional faint band at 70 kDa (Figure 3, right panel, lane A).

Disruption of microsomal membrane integrity with 0.3% CHAPS does not significantly alter the binding of a high-affinity glucose-6-phosphate translocase inhibitor to the high-affinity binding site [15]. Therefore the protein components of glucose-6-phosphate translocase responsible for the high-affinity binding of chlorogenic acid derivatives should be retained after stripping the membranes obtained by washing microsomes with 0.3% CHAPS. Photoaffinity labelling of CHAPS-stripped microsomes from human liver resulted in the labelling of membrane proteins of molecular mass 31, 33, 55 and 70 kDa (Figure 3, right panel, lane B) and 31, 33, 50 and 70 kDa in CHAPS-treated rat liver microsomes (Figure 3, right panel, lane C). Fluorographic detection showed that the labelling pattern of

Figure 5 Phase-separation experiments of rat liver microsomes after photoaffinity labelling with [^3H]S 0957

Rat liver microsomes (600 μg of protein) were incubated for 5 min at 20 $^{\circ}\text{C}$ with 66 nM (12.5 μCi) [^3H]S 0957 followed by irradiation at 254 nm for 20 s. After being washed, integral and peripheral membrane proteins were separated by treatment with CHAPS or Na_2CO_3 . Lane A, control (150 μg of probe); lane B, pellet after treatment of 150 μg of microsomes with 0.3% CHAPS; lane C, supernatant after treatment of 200 μg of microsomes with Na_2CO_3 ; lane D, pellet after treatment of 200 μg of microsomes with Na_2CO_3 . The positions of molecular mass markers are indicated (in kDa) at the left.

CHAPS-treated human liver microsomes (Figure 3, right panel, lane B) was identical with that of 'untreated' human liver microsomes, with the prominent labelling of a 55 kDa protein (Figure 3, right panel, lane A). The labelling pattern of CHAPS-treated rat liver microsomes displayed high incorporation into a 50 kDa and a weaker labelling of 33 and 31 kDa proteins (Figure 3, right panel, lane C) compared with several bands in 'untreated' rat liver microsomes (see Figure 4, right panel, and Figure 6).

The labelling of several bands in rat liver microsomes by [^3H]S 0957 might suggest the non-specific labelling of microsomal proteins unrelated to the putative transport system T1. Because [^3H]S 0957 carries an aromatic azido function that can be transferred to relatively long-lived nitrene intermediates on photolysis, a non-specific promiscuous labelling of several membrane proteins by diffusion of the reactive nitrene seems possible [33]. The extent of such putative non-specific labelling depends greatly on the protein composition of the membrane preparation. This could explain the different labelling patterns of human and rat liver microsomes by the same photoprobe. The findings (1) that S 0957 is a specific high-affinity inhibitor of the microsomal glucose-6-phosphate translocase function and (2) that [^3H]S 0957 labels in human liver microsomes only polypeptides of molecular mass 55, 33, 31 and possibly 70 kDa suggest that these proteins and proteins of molecular mass 50, 33, 31 and 70 kDa in rat liver are hitherto unidentified protein components of the microsomal glucose-6-phosphate translocase postulated by the substrate-translocase model. To test this further, we performed photoaffinity labelling studies with the use of the radioactively labelled glucose 6-phosphate transport inhibitor S 1743, which can be used as a direct photoaffinity probe. On irradiation with UV, highly reactive radical intermediates are generated, either in the aromatic residues of [^3H]S 1743 or in aromatic amino acid residues of the respective binding proteins, that are capable of

Figure 6 Effect of chlorogenic acid derivatives and glucose 6-phosphate on photoaffinity labelling of intact rat liver microsomes by [^3H]S 0957

Intact rat liver microsomes (150 μg of protein, 0.75 mg/ml) were photoaffinity labelled (by irradiation for 20 s at 254 nm after 5 min incubation at 20 $^{\circ}\text{C}$) with 100 nM (1 μCi) [^3H]S 0957 in 50 mM Hepes/HCl buffer (pH 7.0)/250 mM sucrose with no further addition (C) or in the presence of 50 μM chlorogenic acid (CHL), 50 μM S 3483, 50 μM S 4048 or 150 mM glucose 6-phosphate (G-6-P). After separation of membrane proteins by SDS/PAGE, radioactivity was detected by fluorography. The positions of molecular mass markers are indicated (in kDa) at the left; the molecular masses of the bands are indicated at the right.

covalent cross-linking [34]. Irradiation of rat liver microsomes at 300 or 254 nm in the presence of [^3H]S 1743 led to a strong labelling of polypeptides of molecular mass 57 and 50 kDa and a faint labelling of 70, 33, 31 and 22 kDa proteins (Figure 4, left panels). As expected, the rate of photo-cross-linking by the direct photoaffinity probe [^3H]S 1743 is less than 10% of that observed with the nitrene-generating [^3H]S 0957, making detection by fluorography difficult. Nevertheless SDS gels of rat liver microsomes photolabelled with [^3H]S 1743 were exposed for 15–40 weeks for the fluorographic detection of labelled proteins. Figure 4 (right panel) demonstrates that [^3H]S 1743 was incorporated exclusively into sharp bands of molecular mass 57 and 50 kDa (Figure 4, right panel, lane B). Extended photolysis at 300 nm for 5 min led to the additional labelling of a faint band at 70 kDa and of a 22 kDa band (Figure 4, right panel, lane C). Stripping the rat liver microsomes with CHAPS after photolabelling with [^3H]S 0957 revealed the labelling of polypeptides of 50, 33 and 31 kDa in the remaining 'stripped' membranes. CHAPS washing of rat liver microsomes labelled with [^3H]S 1743 showed polypeptides of 50 kDa and to a smaller extent of 22 kDa in CHAPS-stripped microsomes (results not shown).

To reduce the number of candidates for the T1 transporter, we separated integral from peripheral membrane proteins, because a transporter function for one of the labelled binding proteins is possible only for an integral membrane protein. After photoaffinity labelling of rat liver microsomes with [^3H]S 0957, which resulted in the labelling of 70, 57, 54, 50, 41, 33 and 31 kDa proteins (Figure 5, lane A) and subsequent washing with 0.3% CHAPS or 0.1% (v/v) Triton X-100, the incorporation of label

Figure 7 Photoaffinity labelling of particulate material obtained from the livers of a healthy individual and patients with GSD type 1b

Particulate material (200 μg) obtained from human liver biopsies after homogenization and centrifugation at 100000 g was photoaffinity labelled (by irradiation for 20 s at 254 nm after 5 min of incubation at 20 $^{\circ}\text{C}$) with 200 nM (1.5 μCi) [^3H]S 0957 followed by separation of proteins by SDS/PAGE (100 μg of protein per lane), slicing of the gels and determination of radioactivity. (a) Material from control liver; (b) material from GSD type 1b patient 1; (c) material from GSD type 1b patient 2. The molecular masses of the peaks (in kDa) are indicated. (d) Fluorographic detection: lane A, rat liver microsomes labelled with 100 nM (1 μCi) [^3H]S 0957; lane B, material from control liver; lane C, material from GSD type 1b patient 1; lane D, material from GSD type 1b patient 2. The positions of molecular mass markers are indicated (in kDa) at the left.

was seen only into polypeptides of molecular mass 54 and 50 kDa, and to a smaller extent 41, 33 and 31 kDa in the membrane material (Figure 5, lane B). After treatment with Na_2CO_3 at pH 10.5 to remove peripheral membrane proteins, the 70 and 57 kDa proteins were found exclusively in the supernatant fraction (Figure 5, lane C), whereas the 50 kDa and possibly the 54 kDa protein were enriched in the particulate material (Figure 5, lane D). Similar results were obtained with Triton X-114 (results not shown). Because the specific T1 inhibitor [^3H]S 1743 labelled a 50 kDa and a 57 kDa protein nearly exclusively (see Figure 4) and because phase separation experiments with microsomes labelled by [^3H]S 1743 also revealed the identity of the 50 kDa protein as an integral membrane protein and that of the 57 kDa protein as a peripheral membrane protein, the 50 kDa protein in rat liver and its 55 kDa counterpart in human liver are

the most probable candidates for the glucose 6-phosphate transporter T1 in the endoplasmic reticulum membrane.

Specificity of photoaffinity labelling of microsomal proteins by chlorogenic acid derivatives

The specificity of labelling by [^3H]S 0957 was demonstrated by differential labelling experiments with rat liver microsomes in the presence of specific inhibitors of glucose-6-phosphate translocase as well as of compounds known not to interfere with the glucose-6-phosphatase system. Chlorogenic acid specifically inhibited T1 with an IC_{50} of 220 μM , whereas the synthetic analogues S 3483 [14] and S 4048 [13] showed IC_{50} values of 0.2 and 0.002 μM respectively. Differential photoaffinity labelling experiments with

Figure 8 Immunodetection of glucose-6-phosphatase after photoaffinity labelling of rat liver microsomes with [^3H]S 0957

Rat liver microsomes (300 μg of protein) were incubated for 5 min at 20 $^{\circ}\text{C}$ in the dark with 62.5 nM (1 μCi) [^3H]S 0957 followed by irradiation at 20 $^{\circ}\text{C}$ for 20 s. After the microsomes had been washed, microsomal proteins (100 μg of protein per lane) were separated on 9% (top panel), 10.5% (middle panel) and 12% (bottom panel) gels. After blotting, immunodetection was performed with antibodies generated against amino acid residues 203–216 (dilution 1:1000) and subsequently the blots were cut into 2 mm pieces and radioactivity was determined by liquid-scintillation counting after the digestion of proteins with Biolute S. The molecular masses of the peaks (in kDa) are indicated.

[^3H]S 0957 led to a similar ranking of inhibition of labelling of the S 0957-binding proteins to that for inhibition of transport activity (Figure 6). Compounds such as the bile acids cholate and taurocholate, D-glucose, sodium pyrophosphate or sodium phosphate had no effect on the labelling pattern, whereas the anion-transport inhibitor 4,4'-di-isothiocyanostilbene-2,2'-disulphonate (DIDS), previously shown to inhibit microsomal glucose-6-phosphatase [35], decreased the labelling of all bands. The labelling of the 50 and 57 kDa proteins by [^3H]S 1743 was inhibited by chlorogenic acid analogues, the inhibitory potency on photoaffinity labelling showing an identical ranking to that of the inhibition of glucose 6-phosphate transport: S 4048 > S 3483 > chlorogenic acid (results not shown). Glucose 6-phosphate inhibited the labelling of the 33 kDa polypeptide as well as that of the 70, 41 and 31 kDa polypeptides, whereas the labelling of the 50 kDa band seemed unaffected or slightly increased. With

[^3H]S 1743 as a photoaffinity probe, the labelling of the 50 kDa protein was also not decreased significantly by the presence of glucose 6-phosphate. The failure of a ligand to inhibit the labelling of its specific binding protein does not exclude a specific binding of this ligand to its binding protein. As well as other factors, the exchange rates of competing ligands and of the photoprobe for the binding proteins determine the efficiency of photoaffinity labelling and the inhibitory effect of competing ligands, with the possible outcome that a competitive inhibitor to the photoprobe might not lead to an inhibition in the extent of labelling of the respective binding proteins [36]. Photoaffinity labelling in the frozen state allows a snapshot picture of the interaction of a ligand with its binding proteins because all molecular movements and ligand-protein interactions are fixed on shock-freezing [37,38]. Photoaffinity labelling of rat liver microsomes with [^3H]S 0957 or [^3H]S 1743 in the frozen state revealed that the intensity of labelling of the 50 kDa protein was also increased by the presence of glucose 6-phosphate, indicating an increased number of T1-inhibitor-binding sites and/or increase in affinity for T1 inhibitors. These findings are in accordance with the results of Countaway [39] that the incorporation of [^3H]H₂DIDS (an inhibitor of T1 [35]) is increased instead of being decreased by glucose 6-phosphate. It is unclear at present why glucose 6-phosphate increases the number of binding sites accessible to T1-inhibitors.

Photoaffinity labelling of liver tissue samples from GSD type 1b patients

The results from our labelling studies with microsomes from human and rat liver with the use of the photoreactive T1 inhibitors [^3H]S 0957 and [^3H]S 1743 reveal a 55 kDa protein in human liver and a 50 kDa protein in rat liver as the prime candidates for the microsomal glucose 6-phosphate transporter T1. The DNA sequence of a putative glucose-6-phosphate translocase was recently reported on the basis of sequence analysis of this particular gene in two patients with GSD type 1b. In both liver probes, mutations were found in the cDNA sequence of an integral membrane protein with 21–25% similarity to bacterial hexose phosphate transporters [22]. In patient 1, a point mutation Gly³³⁹ → Cys as well as a mutation replacing Glu³⁵⁵ by a stop codon was found, whereas in patient 2 only a point mutation Gly³³⁹ → Cys was reported [22]. A recent publication in which the amino acid sequence of the putative glucose-6-phosphate translocase was analysed in 22 patients with diagnosed GSD type 1b or type 1c revealed mutations in this gene, strengthening its putative role as glucose-6-phosphate translocase [40]. However, the functional link between the gene product and the transport of glucose 6-phosphate has not yet been reported. To investigate the relationship between the T1 candidate proteins identified by photoaffinity labelling and the putative glucose-6-phosphate translocase gene [22], we performed photoaffinity labelling of particulate material obtained from livers of healthy individuals and from patients with diagnosed GSD type 1b. The latter probes were a gift from Professor E. Van Schaftingen. Because the preparation of microsomes was not possible from the small samples of tissue, the available biopsy samples were homogenized and a crude membrane preparation containing all particulate material (membranes and organelles) was prepared and subjected to photoaffinity labelling. The incorporation of label into particulate material prepared from 40 mg of the human control liver resulted in the labelling of 55, 33 and 31 kDa proteins in a pattern similar to that observed with purified microsomes from human liver (Figure 7a). Labelling of an analogous particulate protein fraction isolated from the liver

biopsy of a GSD type 1b patient (patient 1) revealed the absence of the 55 kDa protein and the labelling of the 31 kDa protein only (Figure 7b). Photoaffinity labelling of the particulate material obtained from a second patient with diagnosed GSD type 1b (patient 2), however, led to an identical labelling pattern to that obtained from control liver (Figure 7c). Fluorographic detection demonstrated the prominent labelling of a 55 kDa protein and a faint labelling of 33, 31 and 22 kDa proteins in the particulate fraction of liver homogenate from a healthy individual (Figure 7d, lane B). No 55 kDa protein was detectable in the sample from patient 1 (Figure 7d, lane C), whereas in that from patient 2 a strong labelling of the 55 kDa protein appeared with additional diffuse labelling of 57–60 and 70 kDa bands as well as of the 33 and 31 kDa bands, which were labelled faintly (Figure 7d, lane D). Differential photoaffinity labelling of particulate material from human liver biopsy samples revealed that the labelling of the 55 kDa band was inhibited by the T1 inhibitors and DIDS.

To establish unequivocally the identities of the photolabelled 50 or 55 kDa proteins in rat or human liver respectively with the protein product of the putative glucose-6-phosphatase gene, we prepared antibodies against the epitope of residues 203–216 of the homologous rat protein (P. Habermann, unpublished work). These antibodies generated in rabbits specifically recognized a 50 kDa polypeptide in rat liver microsomes on SDS gels. The addition of the antibodies to solubilized rat liver microsomes did not result in the precipitation of an antigen–antibody complex. We therefore performed photoaffinity labelling experiments of rat liver microsomes followed by blotting and immunodetection. Figure 8 shows that the immunoreactive 50 kDa band comigrated exactly with the radiolabelled 50 kDa polypeptide, independently of the gel concentration used. These results therefore identify the two proteins of molecular mass 50 kDa in the rat and 55 kDa in the human, which were identified by photolabelling with specific T1 inhibitors, as the putative glucose-6-phosphate translocase that was revealed at the cDNA level. Accordingly, the functional identity of the gene product as the glucose-6-phosphate transport protein is now established.

DISCUSSION

The important role of the liver in the regulation of blood glucose levels has stimulated much research on the molecular mechanism of hepatic glucose generation. The key step in hepatic glucose production is catalysed by the glucose-6-phosphatase system in the endoplasmic reticulum of the parenchyma. Despite extensive research efforts since the identification in 1951 of glucose-6-phosphatase activity in the endoplasmic reticulum of hepatocytes [41], the molecular mechanism and structure of this physiologically important enzyme system are far from clear. A major advance was derived from the cloning of murine and human glucose-6-phosphatase by Chou and co-workers [20,42] and the finding that the glucose-6-phosphatase gene is normal in patients suffering from glycogen storage disease types 1b and 1c [43]. Progress on a parallel path came with the development of a new class of highly specific high-affinity inhibitors of the glucose-6-phosphatase system that act exclusively on microsomal glucose 6-phosphate transport while leaving the enzymic phosphohydrolase activity unchanged [12–18]. Thus there now is convincing molecular evidence in favour of the substrate transport model of glucose-6-phosphatase. Moreover, the chlorogenic acid analogues provide new tools for the investigation of the protein components of the glucose 6-phosphate transport system.

Therefore we have synthesized photolabile derivatives of these inhibitors to identify the protein components of the glucose-6-

phosphate translocase by photoaffinity labelling. The photolabile chlorogenic acid analogues S 0957 and S 1743 specifically inhibited the glucose-6-phosphate translocase in microsomes from rat and human liver with IC_{50} values of 160 and 86 nM respectively. Photoaffinity labelling with the nitrene-generating [3H]S 0957 resulted in the labelling of several polypeptides of molecular mass 70, 57, 54, 50, 33 and 31 kDa in rat liver microsomes, whereas in human liver microsomes a polypeptide of molecular mass 55 kDa was predominantly labelled instead of the 50 kDa polypeptide. The direct photoaffinity probe [3H]S 1743 led to the strong labelling of a 57 and 50 kDa polypeptide in rat liver microsomes and a 57 or 55 kDa polypeptide in human liver microsomes. The polypeptides of molecular mass 50, 33 and 31 kDa in the rat and 55, 33 and 31 kDa in human liver were identified as integral membrane proteins, a prerequisite for a membrane protein to act as a solute transporter. Destruction of microsomal membrane integrity with a low concentration of detergents such as 0.3 % CHAPS or 0.1 % Triton X-100 did not significantly alter the specific high-affinity binding of chlorogenic acid derivatives to the glucose-6-phosphate translocase [15]. The photolabelling of these detergent-treated microsomes resulted in the nearly exclusive labelling of polypeptides of molecular mass 50, 33 and 31 kDa in rat liver and 55, 33 and 31 kDa in human liver, making these proteins prime candidates for the glucose-6-phosphate translocase. Three lines of evidence support the identification of the integral membrane proteins of molecular mass 50 kDa in the rat and 55 kDa in humans as the glucose-6-phosphate translocase: first, [3H]S 1743 labels the 50 or 55 kDa protein almost exclusively; secondly, [3H]S 1743 is a high-affinity inhibitor of glucose-6-phosphate translocase; and thirdly, [3H]S 1743 generates short-lived highly reactive intermediates on UV irradiation, with a low probability of non-specific protein labelling. Antibodies generated against residues 203–216 of the rat protein specifically recognized the photolabelled 50 kDa protein in rat liver microsomes. The reason for the slight molecular mass difference between the human and rat proteins is not clear. Integral membrane proteins often show different apparent molecular masses on SDS/PAGE despite high sequence homology; for example, the Na^+ /bile acid co-transporters from rat and rabbit show nearly 90 % homology but on SDS gels the dimeric transporters show apparent molecular masses of 99 and 93 kDa respectively [38].

During the preparation of this paper the cDNA sequence of a putative glucose-6-phosphate translocase was reported, supported by the finding that the gene was mutated in two patients with clinically and biochemically diagnosed GSD type 1b [22]. However, the functional identity of the protein product of this gene remained to be established. This link has been established in the present study. Professor Van Schaftingen generously supplied us with the biopsy samples of these patients to perform photoaffinity labelling studies. Even in crude membrane fractions a prominent labelling of the 55 kDa polypeptide was seen in the biopsy sample of healthy individuals. The 55 kDa polypeptide, however, was not labelled in the liver sample of patient 1. In the protein from patient 1, a point mutation Gly³³⁹ → Cys as well as a mutation replacing Glu³⁵⁵ by a stop codon was found in the putative glucose-6-phosphate translocase gene [22]. These mutations would remove the terminal two transmembrane helices as well as the targeting signal for retention of the protein in the endoplasmic reticulum. The failure to label the 55 kDa protein in the particulate fraction from the liver of this patient suggests the absence of the 55 kDa protein from the endoplasmic reticulum. In the liver sample of patient 2, however, the 55 kDa protein was labelled by the chlorogenic acid photoprobes. In the protein from patient 2, only a point mutation Gly³³⁹ → Cys was found in

the putative glucose-6-phosphate translocase gene [22], conserving the C-terminus with its retention signal for the endoplasmic reticulum. The diagnosis of GSD type 1b phenotype and the labelling of the 55 kDa protein suggests a functional impairment of the glucose 6-phosphate transporter protein in this patient. Taken together, the studies in the present paper strongly support the identity of the 55 kDa protein as the human microsomal glucose-6-phosphate translocase.

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