Volume 229, number 1, 179–182 FEB 05628 February 1988

The identification of T_2; the phosphate/pyrophosphate transport protein of the hepatic microsomal glucose-6-phosphatase system

Ian D. Waddell, J. Gordon Lindsay* and Ann Burchell

Department of Medicine, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY and **Department of Biochemistry, University of Glasgow, Glasgow G12 8QQ, Scotland*

Received 28 December 1987

The phosphate/pyrophosphate translocase protein (T_2) of the hepatic microsomal glucose-6-phosphatase system was identified and then purified using antibodies raised against the rat mitochondrial phosphate/hydroxyl ion antiport protein. The T_2 protein was shown to be absent in the microsomes isolated from a patient previously diagnosed as having type 1c glycogen storage disease.

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

1. INTRODUCTION

Hepatic microsomal glucose-6-phosphatase (EC 3.1.3.9) catalyses a key step in the homeostatic regulation of blood glucose levels [1]. Substantial kinetic [2] and genetic evidence [3–5] indicates that glucose 6-phosphate hydrolysis is catalysed by a multicomponent system comprised of the glucose-6-phosphatase enzyme with its active site situated in the lumenal surface of the endoplasmic reticulum and three translocases which facilitate movement of glucose 6-phosphate (T_1), phosphate and pyrophosphate (T_2) and glucose (T_3) between the cytosol and the lumen of the endoplasmic reticulum. The absence of any of these proteins will impair glucose 6-phosphate hydrolysis and cause type 1a, 1b, 1c or 1d glycogen storage disease, respectively [5]. However, none of the transport proteins of the glucose-6-phosphatase system have been unequivocally identified or purified.

Here, we identify and purify T_2 the hepatic microsomal phosphate/pyrophosphate translocase using antibodies raised against the rat mitochondrial phosphate/hydroxyl ion antiport protein [6] and demonstrate that the hepatic microsomal T_2 protein is absent in an adult patient previously diagnosed as having type 1c glycogen storage disease [5].

Correspondence address: A. Burchell, Department of Medicine, Ninewells Hospital and Medical School, University of Dundee, Dundee DD1 9SY, Scotland

2. MATERIALS AND METHODS

Biotinylated anti-rabbit antibody and streptavidin-linked peroxidase complex were purchased from Amersham (Amersham, England). 4-Chloro-1-naphthol, glucose 6-phosphate (monosodium salt), mannose 6-phosphate (monosodium salt) and prestained molecular mass markers were obtained from Sigma (Poole, England). Empigen BB was the kind gift of Albright and Wilson (Whitehaven, England). Nitrocellulose was obtained from Schleicher and Schuell (Dassell, FRG) and CNBr-activated Sepharose from Pharmacia (Uppsala). All other reagents were analytical reagent grade.

2.1. *Sample preparation*

Microsomes [6] and mitochondria [7] were prepared from the livers of starved Wistar rats as described. Samples of microsomes and mitochondria were prepared from fresh human liver biopsy samples as in [5]. Plasma membrane fragments were removed from preparations using agarose-bound Concanavalin A as in [8]. Details of the patient with type 1c glycogen storage disease and control cases were also given in [5]. Ethical approval was given by the Tayside Health Board Ethical Committee.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

2.3. *Assay of glucose-6-phosphatase*

Glucose-6-phosphatase and mannose-6-phosphatase activities were assayed as in [9] and are expressed as μmol P_i released/min per mg microsomal protein. Protein concentrations were measured by the method of Peterson [10].

2.3. *Antisera*

Polyclonal antisera to the rat liver mitochondria phosphate/hydroxyl ion antiport protein was raised in rabbits as described [7]. IgG was further purified by $(NH_4)_2SO_4$ fractionation as in [11]. Mitochondrial phosphate/hydroxyl ion antiport protein antibody-Sepharose columns were prepared using CNBr-Sepharose according to [12].

2.4. *Immunoblot analysis*

Microsomal or mitochondrial samples were subjected to electrophoresis on a 7–16% polyacrylamide gel as described by Laemmli [13] and silver stained [14] or electrophoretically transferred to nitrocellulose as in [15] or in the presence of 1% Empigen BB [16] and visualised with antimitochondrial phosphate/hydroxyl ion antiport protein antibodies, a biotinylated anti-rabbit second antibody and a streptavidin-linked peroxidase complex as the detection system using 4-chloro-naphthol as substrate. The molecular masses of the Sigma prestained standard proteins on the immunoblots were 180, 116, 84, 58, 48.5, 36.5 and 26.6 kDa. The molecular masses of the Sigma standard proteins on the silver-stained gel were 66, 45, 36, 29, 24, 20.1 and 14.2 kDa.

2.5. *Purification of T_2*

Fresh microsomes were eluted from a Concanavalin A column and extracted in buffer A (0.5% Triton X-100, 20 mM LiCl, 0.1 mM EDTA, 0.5 mM dithiothreitol, 20 mM phosphate, pH 7.0) and centrifuged at 105000 × *g* for 1 h. The pellet was resuspended in buffer A plus 8% Triton X-100 and centrifuged at 105000 × *g* for 1 h. The supernatant was run through a Sephadex G-25M column to remove excess dithiothreitol before being applied to a phosphate/hydroxyl ion antiport protein antibody Sepharose column. After 1 h unbound protein was washed off the column and the affinity-purified protein eluted by 1 M glycine, pH 4.0.

3. RESULTS AND DISCUSSION

3.1. *Identification of T_2*

T_2, the phosphate/pyrophosphate transport protein of the hepatic microsomal glucose-6-phosphatase system has not been identified or purified nor are any of the known inhibitors of T_2 [17] specific enough to be used to identify the protein. Here, we have used antibodies raised against another phosphate transport protein, the rat hepatic mitochondrial phosphate/hydroxyl ion transport protein, to identify T_2.

The rabbit antibody to the mitochondrial phosphate/hydroxyl ion antiport protein was shown to be a specific antibody as it only cross-reacts with one polypeptide in immunoblot analysis of control human hepatic mitochondria (fig.1, lanes 4,5) and hepatic mitochondria from patients with either type 1b or type 1c glycogen storage disease (fig.1, lanes 2,3). In similar immunoblots of hepatic microsomes no cross-reacting bands were visualised (not shown). In these initial immunoblotting studies proteins were transferred from SDS-polyacrylamide gels to nitrocellulose essentially as described by Towbin et al. [15] and the proteins would therefore have been in a denatured state on the nitrocellulose filters. These results therefore indicate that antibodies

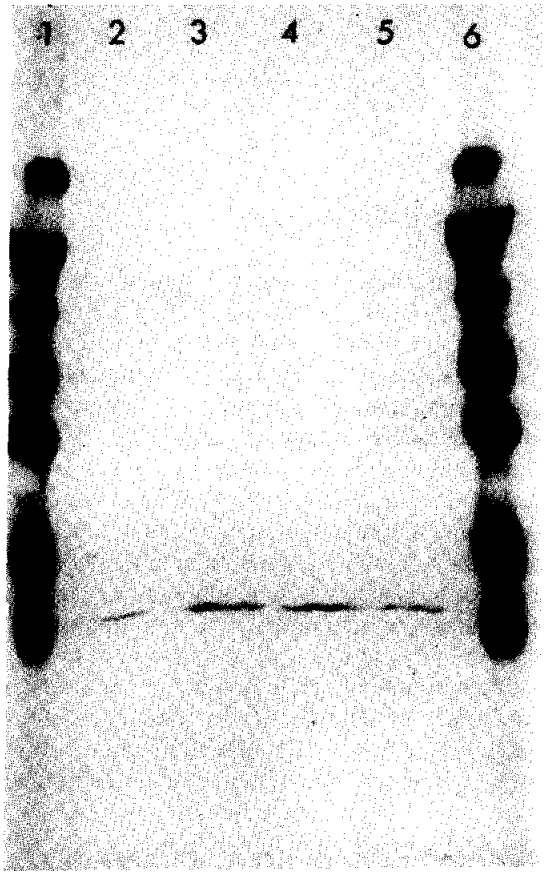

Fig.1. Immunoblot analysis of human hepatic mitochondria. Lanes: 1,6, prestained Sigma protein molecular mass standards; 2, human hepatic mitochondria from a patient with type 1b glycogen storage disease (20 μg); 3, human hepatic mitochondria from a patient with type 1c glycogen storage disease (20 μg); 4,5, control human hepatic mitochondria (20 μg).

raised to the mitochondrial phosphate transport protein do not cross-react with the denatured T_2 protein.

However, as both proteins transport phosphate it seemed possible that they might have some secondary structure similarities. Microsomal proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose in the presence of 0.1% of the zwitterionic detergent Empigen BB as described in [16] to allow partial renaturation of the proteins. Under these renaturing conditions the antibody cross-reacted with a single polypeptide in both rat (fig.2, lane 1) and human hepatic microsomes from control patients (fig.2, lanes 4,5,7). In contrast, the antibody's reaction with the mitochondrial phosphate/hydroxyl ion antiport protein becomes much weaker after partial renaturation of the protein with Empigen BB (not shown).

The identification of the immunoreactive polypeptide in microsomes as T_2 was confirmed by the complete absence of a cross-reacting protein in the hepatic microsomes isolated from an adult patient previously diagnosed [5] as having type 1c glycogenosis which is caused by a deficiency to T_2 (fig.2, lane 6). The type 1c glycogen storage patient had been shown (fig.1, lane 3) to have normal levels of the hepatic mitochondrial phosphate/hydroxyl ion antiport protein, confirming that the cross-reacting band in microsomes was not due to

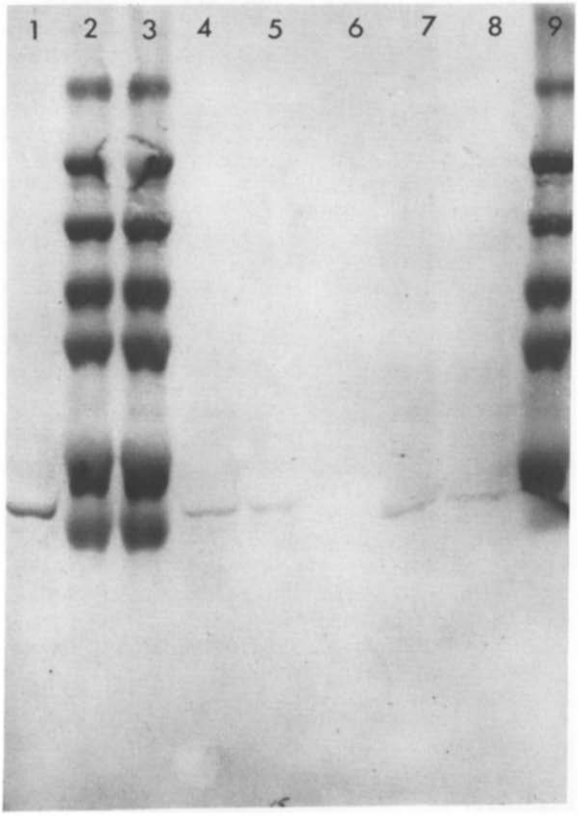

Fig.2. Immunoblot analysis of rat and human hepatic microsomes. Lanes: 1, rat hepatic microsomes (10 μg); 2,3,9, prestained Sigma protein molecular mass standards; 4,5,7, control human hepatic microsomes (20 μg); 6, human hepatic microsomes from a patient with type 1c glycogen storage disease (20 μg); 8, human hepatic microsomes from a patient with type 1b glycogen storage disease (20 μg).

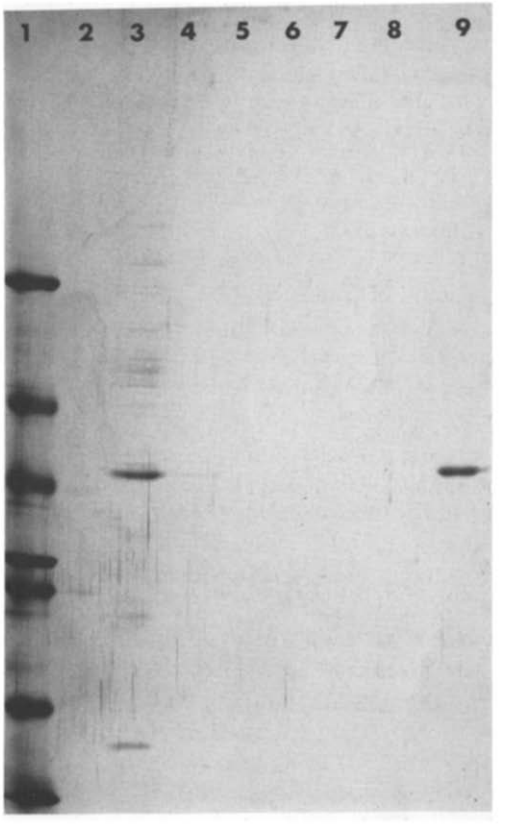

Fig.3. SDS-polyacrylamide gel electrophoresis of purified rat hepatic microsomal T_2. Lanes: 1, Sigma molecular mass standard proteins; 2–4, breakthrough from phosphate/hydroxyl ion antiport protein antibody Sepharose column; 5–9, fractions eluted from a phosphate/hydroxyl ion antiport protein antibody Sepharose column at pH 4.0.

the presence of a mitochondrial contamination. The microsomal phosphate transport protein was also shown to be present in a patient with glycogen storage disease type 1b which is a deficiency of T_1, the hepatic microsomal glucose 6-phosphate transport protein confirming that T_1 and T_2 are indeed different proteins.

The subunit molecular mass of T_2 is 37 kDa as judged by SDS-polyacrylamide gel electrophoresis (described in section 2). However, the apparent molecular mass varies somewhat depending on both the acrylamide concentration of the gel and the ionic strength of the buffer which suggests that T_1 might be a glycoprotein. Unequivocal determination of the exact molecular mass of T_2 must therefore await the determination of the sequence of the characterised protein. In contrast, the mitochondrial phosphate/hydroxyl ion antiport protein has been shown not to be a glycoprotein and has a molecular mass of 34 kDa [7].

3.2. *Purification of T_2*

The ability to detect the T_2 protein using immunoblotting made it possible to devise a purification scheme for T_2. T_2 is an integral membrane protein and is not easily solubilised from the microsomal membrane. Rat hepatic microsomes were therefore extracted in 0.5% Triton X-100 (as detailed in section 2) which removes a large number of microsomal proteins and then 8% Triton X-100 was used to solubilise T_2. The partially purified T_2 was then applied to a mitochondrial phosphate/hydroxyl ion and part protein antibody Sepharose column. The T_2 protein was eluted with 1 M glycine, pH 4.0, and was completely homogeneous as judged by silver staining of SDS-polyacrylamide gels (fig.3). The purified protein is currently being used to raise polyclonal antiserum to T_2 in sheep.

The identification and purification of T_2, the phosphate/pyrophosphate transport protein, have provided the necessary tools to approach the isolation of DNA probes for the protein which are required to allow the prenatal diagnosis of type 1c glycogen storage disease.

Acknowledgements: The authors wish to thank Miss Lesley Stewart for excellent technical assistance and Dr Brian Burchell for the suggestion that antibody to the rat hepatic mitochondrial phosphate/hydroxyl ion antiport protein could interact with T_2. This work was supported by grants from the Scottish Hospitals Endowment Research Trust and the British Diabetic Association to A.B.

REFERENCES

[1] Ashmore, J. and Weber, G. (1959) Vitam. Horm. 18, 91–133.
[2] Arion, W.J., Lange, A.J., Walls, H.E. and Ballas, L.M. (1980) J. Biol. Chem. 255, 10396–10406.
[3] Lange, A.J., Arion, W.J. and Beaudet, A.L. (1980) J. Biol. Chem. 255, 8381–8384.
[4] Nordlie, R.C. (1985) Trends Biochem. Sci. 10, 70–75.
[5] Burchell, A., Jung, R.T., Lang, C.C., Bennet, W. and Shepherd, A.N. (1987) Lancet 1, 1059–1062.
[6] Arion, W.J., Burchell, B. and Burchell, A. (1984) Biochem. J. 220, 835–842.
[7] Gibb, G.M., Reid, G.P. and Lindsay, J.G. (1986) Biochem. J. 238, 543–551.
[8] Sehmi, M., Williams, L.G. and Williams, D.J. (1982) Biochem. Soc. Trans. 10, 37.
[9] Burchell, A., Hume, R. and Burchell, B. (1987) Clin. Chim. Acta, in press.
[10] Peterson, G.L. (1977) Anal. Biochem. 83, 346–356.
[11] Burchell, A. and Cain, D.I. (1985) Diabetologia 28, 852–856.
[12] Cohen, P.T.W., Burchell, A. and Cohen, P. (1976) Eur. J. Biochem. 66, 347–356.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Oakley, B.R., Kirslm, D.R. and Morris, N.R. (1980) Anal. Biochem. 105, 361–363.
[15] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[16] Mandrell, R.E. and Zollinger, W.D. (1984) J. Immun. Methods 67, 1–11.
[17] Arion, W.J., Lange, A.J. and Walls, H.E. (1980) J. Biol. Chem. 255, 10387–10395.