Membrane-bound *N*-acetyl-β-glucosaminidase

Different binding specificity in control and I-cell disease livers

Goro Kato* and Yoshiyuki Suzuki+

Division of Chemical Pathology, National Center for Nervous, Mental and Muscular Disorders, Ogawa-Higashimachi, Kodaira, Tokyo 187 and +*Department of Pediatrics, Faculty of Medicine, The Universty of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan*

Received 30 August 1985

Several lysosomal enzymes solubilized at pH 4 from saponin-treated membranes showed markedly variable affinities to the bovine liver phosphomannosyl receptor in both bovine and human livers. The enzymes from I-cell disease liver did not bind the receptor in spite of normal intracellular activities. *N*-Acetyl-β-glucosaminidase was effectively released from the membrane preparation of a control liver by mannose 6-phosphate, and other sugars showed little effect in this experiment. However, in the I-cell disease liver, dissociation occurred not by mannose 6-phosphate but by other sugars, such as fucose, mannose and *N*-acetyl-D-glucosamine. These results indicate the presence of an alternate transport system other than the pathway mediated by the mannose 6-phosphate receptor, and the role of other sugar-binding proteins is discussed in intracellular processing and transport of newly synthesized enzymes.

Phosphomannosyl receptor *Lysosomal enzyme* *Intracellular transport* *Alternate pathway* *I-cell disease*

1. INTRODUCTION

Lysosomal enzymes are glycoproteins with high mannose type oligosaccharides in their molecules. Mannose 6-phosphate has been found in these sugar chains and characterized as a specific recognition marker of these enzymes in mammalian cells. Recent studies revealed that the phosphomannosyl receptor (PMR) binds and transports enzyme molecules to lysosomes [1]. PMR was purified from some mammalian tissues [2–6]. However, in previous studies the binding activity was investigated on soluble 'high-uptake' enzymes obtained from materials different from those for preparation of the receptor [2–5]. The in vivo process of binding and transport of enzymes is not yet clear.

Recently the mouse P338D$_1$ macrophage was found to show high levels of intracellular acid hydrolase activities in spite of deficient PMR [7]. Furthermore, activities of lysosomal enzymes were normal in tissues of I-cell disease, a human disease caused by a defect in phosphorylation of these enzyme molecules [8,9]. These results suggested the presence of an alternate pathway for the intracellular transport of lysosomal enzymes other than the PMR system.

We report here different patterns in the binding of several lysosomal enzymes in hepatic membrane preparations to the bovine liver PMR. Among those, the pattern in the dissociation of the membrane-bound *N*-acetyl-β-glucosaminidase induced by various sugars showed definite changes in I-cell disease.

* To whom correspondence should be addressed, at: Department of Biochemistry, Yamanashi Medical College, Tamahomachi, Nakakoma, Yamanashi 409-38, Japan

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

2. MATERIALS AND METHODS

2.1. *Tissues*

Bovine liver tissues obtained from a slaughterhouse were kept frozen at −70°C, and human liver autopsy specimens were kept frozen at −70°C (control) or −20°C (control and I-cell disease) until use. The diagnosis of I-cell disease was established in a 2-month-old boy who presented with severe somatic dysmorphism, typical inclusion bodies in fibroblasts and changes in intracellular and extracellular activities of lysosomal enzymes.

2.2. *Purification of bovine liver PMR and preparation of anti-PMR antibody*

PMR was extracted and purified from fresh bovine liver membranes, and the anti-PMR antibody was prepared and purified as described [10].

2.3. *Preparation of acid-soluble enzymes (ASE) from the saponin-treated total membrane fraction*

The liver homogenate was prepared in 50 mM Tris-HCl (pH 7.0) containing 0.25 M sucrose (1 g in 10 ml) with a Potter-Elvehjem type homogenizer at 900 rpm (5 strokes) twice at 4°C. The homogenate was centrifuged at $800 \times g$ for 5 min and the supernatant at $105\,000 \times g$ for 1 h. The precipitate (TMF) was suspended in 8 ml of 50 mM citrate-phosphate buffer (pH 7.0) containing 0.25 M sucrose and 0.5% saponin (CPSS buffer), and then incubated at 4°C for 30 min. The suspension was centrifuged and the precipitate (saponin-treated TMF) washed repeatedly in CPSS buffer until no further decrease in enzyme activities was observed in the supernatant. Finally the precipitate was suspended in 8 ml CPSS buffer (pH 4.0), and incubated at 4°C. After 30 min the suspension was centrifuged and the supernatant (ASE fraction) stored at −20°C.

2.4. *Preparation of immobilized PMR*

PMR (0.5 μg) and anti-PMR antibody (3 μg) in 30 μl PBS (20 mM potassium phosphate, 0.15 M NaCl, 0.02% NaN_3, pH 7.2) were mixed with 20 μl of 20% (v/v) IgG Sorb (Enzyme Center). The mixture was incubated at 37°C for 20 min, and then added to 50 μl Tris buffer (50 mM Tris, 0.05% Triton X-100, 0.15 M NaCl, 5 mM NaH_2PO_4, 0.025% NaN_3, pH 7.5) or 50 μl of 60 mM mannose 6-phosphate in the same buffer.

2.5. *Assays*

Enzyme activities were determined using 4-methylumbelliferyl glycosides (Nakarai) as substrates at 37°C in 60 μl of 0.2 M citrate/0.2 M NaCl (pH 4.5). Reactions were stopped by addition of 0.7 ml of 0.5 M glycine carbonate, pH 10.5. One unit of activity was defined as 1 nmol 4-methylumbelliferone liberated per h. Protein was determined by the method of Lowry et al. [11].

3. RESULTS

3.1. *Binding of ASE to immobilized bovine liver PMR*

The rate of binding was calculated from the amounts of added and bound ASE, and is summarized in fig.1. In bovine liver, the majority (about 80%) of α-mannosidase and β-glucuronidase activities was found to bind the PMR, while binding occurred in less than 10% of α-glucosidase and α-galactosidase activities, and no significant binding was found in β-glucosidase activity. Other enzymes showed intermediate rates of binding. In the human liver frozen at −70°C for 7 months, measurable activities of *N*-acetyl-β-glucosaminidase, β-glucuronidase and α-glucosidase were detected in the ASE fraction. The rates of binding in these human enzymes were similar to those in bovine enzymes: β-glucuronidase, 80%; *N*-acetyl-β-glucosaminidase, 50%; α-glucosidase, 2%. Almost identical results were also demonstrated in another control specimen frozen at −20°C for several years. However, β-glucuronidase and *N*-acetyl-β-glucosaminidase in the ASE fraction of the liver from a patient with I-cell disease did not bind PMR although activities of these enzymes were normal.

3.2. *Release of membrane-bound enzymes by various sugars*

Mannose 6-phosphate (10 mM) was most effective for dissociation of lysosomal enzymes from the saponin-treated TMF in the bovine liver; 70% of the activity in the ASE fraction was released for β-glucuronidase, 27% for α-glucosidase, 60% for β-galactosidase, 43% for *N*-acetyl-β-glucosaminidase, 50% for α-fucosidase, and 90% for α-

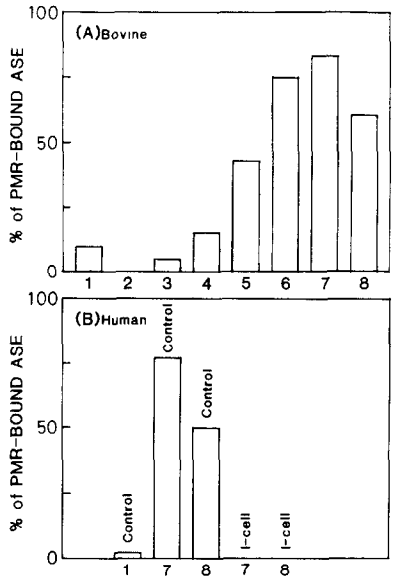
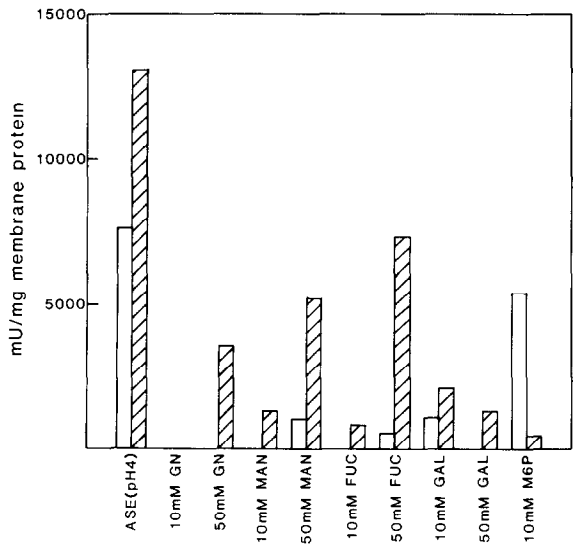


Fig.1. Percentage of bovine and human liver ASE bound to immobilized PMR. The ASE fraction (200 μl) diluted appropriately with Tris buffer containing 0.2% BSA (Tris-BSA) was added to 50 μl immobilized PMR suspension in the presence or absence of mannose 6-phosphate. The mixture was shaken and incubated for 90 min at 4°C. The ASE-PMR complex was collected by centrifugation at 1500×g for 10 min and the pellet washed 4 times with Tris-BSA. Finally the pellet was suspended in 40 μl of 0.2 M citrate/0.2 M NaCl (pH 4.5), incubated at 37°C for 30 min, and centrifuged. Activities of lysosomal enzymes were assayed in the supernatant. The amount of each enzyme bound to immobilized PMR in the presence of mannose 6-phosphate was subtracted from that in the absence of mannose 6-phosphate. The final washing solution of saponin-treated TMF before extraction of ASE was also subjected to the same binding assay procedure. Finally the following calculation was performed.

$$\text{corrected rate of binding} = \frac{A_b - S_b}{A_a - S_a} \times 100,$$

where A_a = the enzyme activity added to the immobilized PMR in the ASE fraction, A_b = the enzyme activity bound, S_a = the enzyme activity added to the immobilized PMR in the final washing solution of the saponin-treated TMF, and S_b = the enzyme activity bound. Each value represents the mean of 2 or 3 separate experiments. The corrected values of added and bound enzymes are as follows. (A) Bovine liver: (1) α-glucosidase, 29 and 2.8 mU; (2) β-glucosidase, 110 and 0 mU; (3) α-galactosidase, 13 and 0.7 mU; (4) β-galactosidase, 67 and 11 mU; (5) α-fucosidase, 140 and 60 mU; (6) α-mannosidase, 20 and 16 mU; (7) β-glucuronidase, 24 and 20 mU; (8) N-acetyl-β-glucosaminidase, 360 and 220 mU. (B) Human control liver: (1) α-glucosidase, 18 and 0.3 mU; (7) β-glucuronidase, 31 and 24 mU; (8) N-acetyl-β-glucosaminidase, 184 and 90 mU. I-cell disease liver: (7) β-glucuronidase, 15 and 0 mU; (8) N-acetyl-β-glucosaminidase, 200 and 0 mU. Specific activities (unit/mg protein) of I-cell disease and control ASE fractions are 6.4 and 5.1 for β-glucuronidase, and 44 and 51 for N-acetyl-β-glucosaminidase, respectively.



←

Fig.2. Displacement of N-acetyl-β-glucosaminidase in saponin-treated TMF by various sugars. This saponin-treated TMF was prepared from control (open bars) and I-cell disease liver (hatched bars), and washed repeatedly with 50 mM Tris-HCl buffer (pH 7.0) containing 0.25 M sucrose and 0.5% saponin. The washed TMF was suspended and incubated for 6 h at 4°C in 8 ml of the buffer containing various sugars or the buffer alone. The suspension was centrifuged and the enzyme activity in the supernatant was assayed. The amount of the enzyme released in the absence of the sugar was subtracted from that in the presence of the sugar. Each value represents the mean of 2 separate experiments.

mannosidase. Other sugars were less effective or not effective. The following amounts of dissociation were observed with 3 different sugars tested; 30% for β-glucuronidase, 21% for α-fucosidase and 21% for *N*-acetyl-β-glucosaminidase with 50 mM mannose; 24% for *N*-acetyl-β-glucosaminidase and 29% for α-mannosidase with 50 mM fucose; 23% for β-glucuronidase with 50 mM galactose.

The amounts of *N*-acetyl-β-glucosaminidase released by various sugars from the saponin-treated human liver TMF are shown in fig.2. In the control liver 10 mM mannose 6-phosphate dissociated the largest amount of the enzyme activity (71% of ASE) from the membrane preparation. The dissociation was lower with other sugars: 14% with 10 mM galactose, 10% with 50 mM mannose and 7% with 50 mM fucose. In contrast, there was little dissociation with mannose 6-phosphate in the I-cell disease liver: 3.4% at 10 mM and 19% at 50 mM. Instead, 50 mM fucose was the most effective (56%), followed by 50 mM mannose (40%) and 50 mM *N*-acetyl-β-glucosamine (27%).

4. DISCUSSION

Lysosomal enzyme proteins are considered to be sorted out by specific receptors after de novo synthesis, and transported to lysosomes. The PMR-mediated transport system has been recognized as one of these systems in human fibroblasts. It is highly likely that the ASE fraction in our study contained mainly these intermediate enzymes, as enzymes were extracted by treatment under acidic conditions from the membrane fraction. Mature enzymes in lysosomes must have been released by the detergent, and those tightly bound to lysosomal membranes in the acidic environment in vivo could not be solubilized further by the procedure of acid extraction. It was demonstrated that the bovine liver PMR had a high affinity to α-mannosidase and β-glucuronidase in bovine liver, and might be the major transporter of these 2 enzymes. Other enzymes were not or only partially bound to the PMR. This result indicated that other PMR components [12,13] or other recognition systems were also working for the transport of many lysosomal enzymes.

PMR also seems to mediate the intracellular transport of newly synthesized lysosomal enzymes in human liver [10]. In some enzymes examined, the pattern of binding to the bovine PMR was similar to that in bovine liver. Consequently, it can be concluded that the PMR system functions as a major transporter for β-glucuronidase in humans as well as in bovines. Other recognition markers may exist for other enzymes. There must be another transport pathway in humans for targeting of β-glucuronidase and *N*-acetyl-β-glucosaminidase, since activities of these enzymes were normal in the ASE fraction of the liver from a patient with I-cell disease in spite of deficient binding to PMR. β-Glucosidase is also expected to be associated with a recognition system other than the PMR pathway because this enzyme in the ASE fraction of bovine liver did not bind PMR and its activity was normal in I-cell disease fibroblasts in humans [14], and because no phosphate was shown in the molecule of human placental β-glucocerebrosidase [15].

What is the molecular structure of the alternate pathway? Some mammalian lectins (sugar-binding proteins) may act as receptors for processing and sorting of carbohydrate chains in glycoprotein molecules [16]. Our data indicated that several enzymes were associated with the TMF preparation in bovine liver, and strongly dependent upon the mannose 6-phosphate recognition system, with the exception of β-glucosidase. Some enzymes were released from TMF by mannose, fucose and galactose. Binding proteins specific to these sugars may exist and participate in the processing and the intracellular transport of lysosomal enzymes.

This study has provided a definite clue to the molecular basis of the alternate pathway. In this experiment dissociation of *N*-acetyl-β-glucosaminidase from TMF was different between I-cell disease and control livers. This enzyme in ASE from the I-cell disease liver did not bind PMR, and the dissociation occurred in the presence of fucose, mannose and *N*-acetyl-D-glucosamine, but little in the presence of mannose 6-phosphate. Probably *N*-acetyl-β-glucosaminidase is bound mostly to PMR under physiological conditions, but in I-cell disease it may be sorted and transported as a glycoprotein with complex-type oligosaccharides, by other binding proteins, such as fucose-binding protein or mannose(*N*-acetyl-D-glucosamine-binding protein [16,17]. Fucose is also known to

exist in high mannose type oligosaccharides [18]. Fucose, mannose and galactose were effective in releasing *N*-acetyl-β-glucosaminidase to some extent in the control liver. These sugars may function as recognition markers for the processing of oligosaccharides and for the intracellular transport of this enzyme, especially when the phosphate marker is deficient in the ligand molecule as observed in I-cell disease [19]. A processing and transport system not known to us at present could have been newly established in this disease under collaboration of binding proteins different from the PMR system. Finally the possibility cannot be ruled out that PMR itself recognizes molecular markers other than mannose 6-phosphate.

ACKNOWLEDGEMENTS

This work was supported by grants from the Ministry of Education, Science and Culture, and the Ministry of Health and Welfare of Japan.

REFERENCES

[1] Sly, W.S. and Fischer, H.D. (1982) J. Cell. Biochem. 18, 67–85.
[2] Sahagian, G.G., Distler, J.J. and Jourdian, G.W. (1981) Proc. Natl. Acad. Sci. USA 78, 4289–4293.
[3] Sahagian, G.G., Distler, J.J. and Jourdian, G.W. (1982) Methods Enzymol. 83, 392–396.
[4] Fischer, H.D., Creek, K.E. and SLy, W.S. (1982) J. Biol. Chem. 257, 9938–9943.
[5] Steiner, A.W. and Rome, L.H. (1982) Arch. Biochem. Biophys. 214, 681–687.
[6] Brown, W.J. and Farquhar, M.G. (1984) Cell 36, 295–307.
[7] Gabel, C.A., Goldberg, D.E. and Kornfeld, S. (1983) Proc. Natl. Acad. Sci. USA 80, 775–779.
[8] Owada, M. and Neufeld, E.F. (1982) Biochem. Biophys. Res. Commun. 105, 814–820.
[9] Waheed, A., Pohlmann, R., Hasilik, A., Von Figura, K., Van Elsen, A. and Leroy, J.G. (1982) Biochem. Biophys. Res. Commun. 105, 1052–1058.
[10] Kato, G. and Suzuki, Y. (1985) J. Appl. Biochem., in press.
[11] Lowry, O.H., Rosebrough, M.J., Farr, A.L. and Randall, R.L. (1951) J. Biol. Chem. 193, 265–275.
[12] Alvares, K. and Balasubramanian, A.S. (1983) Biochem. Biophys. Res. Commun. 112, 398–406.
[13] Mitchell, D.C., maler, T. and Jourdian, G.W. (1984) J. Biol. Chem. 24, 319–330.
[14] Neufeld, E.F., Lim, T.W. and Shapiro, L.J. (1975) Annu. Rev. Biochem. 44, 357–376.
[15] Takasaki, S., Murray, G.J., Furbish, F.S., Brady, R.O., Barranger, J.A. and Kobata, A. (1984) J. Biol. Chem. 259, 10112–10117.
[16] Ashwell, G. and Harford, J. (1982) Annu. Rev. Biochem. 51, 531–554.
[17] Mizuno, Y., Kozutsumi, Y., Kawasaki, T. and Yamashina, I. (1981) J. Biol. Chem. 256, 4247–4252.
[18] Howard, D.R., Natowicz, M. and Baenziger, J.U. (1982) J. Biol. Chem. 257, 10861–10868.
[19] Brown, W.J. and Farquhar, M.G. (1984) Proc. Natl. Acad. Sci. USA 81, 5135–5139.