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THE CONVERSION OF THE PRECURSOR FORM OF γ-GLUTAMYLTRANSPEPTIDASE TO ITS SUBUNIT FORM TAKES PLACE IN BRUSH BORDER MEMBRANES

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SUMMARY: The subcellular distributions of the precursor form and mature form of γ-glutamyltranspeptidase of rat kidney were studied by labeling the enzyme with [^{3}H]fucose *in vivo*. In *trans* Golgi elements and basolateral membranes, γ-glutamyltranspeptidase was present as a precursor form with a single polypeptide chain. However, the brush border membranes contained the heavy and light subunits as well as precursor. These results suggest that the precursor is converted to the mature form after its transport to the brush border membranes.

γ-Glutamyltranspeptidase [EC 2.3.2.2] of rat kidney is a glycoprotein bound to the brush border membranes. It is composed of two nonidentical subunits and the amino-terminal portion of the heavy subunit anchors the enzyme to the membrane (1-5). This enzyme is synthesized as a precursor form with a single polypeptide chain, and the precursor is split posttranslationally into the two subunits of the mature form (6-8). In the previous report, we suggested that [^{3}H]fucose is incorporated into the precursor and that proteolytic processing takes place in the brush border membranes (8). In the present communication, we describe the distributions of labeled precursors among organelles, and conclude that the precursor is split into subunits after its transfer to the brush border membranes.

MATERIALS AND METHODS

Chemicals and animals: Adenosine 5'-triphosphate, ouabain and UDP-galactose were purchased from Sigma Chemical Co.. Ovomucoid was from Boehringer-Mannheim GmbH. L-[5,6-^{3}H]Fucose (45 Ci/mmole) and UDP-D-[6-^{3}H]galactose (16.3 Ci/mmole) were from Amersham. Animals and other chemicals were as described previously (7).

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Assay procedures: γ-Glutamyltranspeptidase was measured as described previously (2). Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as a standard. The activities of total and ouabain-sensitive Na^+, K^+-dependent ATPase were measured by the method of Fujita et al.(10) except that the ouabain concentration in assays was 2.5 mM. UDP-galactose: ovomucoid galactosyltransferase activity was determined by the method of Bretz and Stäubli (11).

[^{3}H]Fucose labeling and subcellular fraction: The procedure was described in detail in the previous paper (8). Rats weighing 160-170 g were starved overnight and then injected intravenously with 250 μCi of [^{3}H]fucose in 1 ml of saline, and killed 10, 30 and 60 min later. In each experiment, organelles were isolated from the kidneys of two rats. The kidney tissue was passed through a stainless-steel strainer, mixed with 20 ml of 0.25 M sucrose solution and homogenized in a Dounce homogenizer. The homogenate was centrifuged at 400 xg for 10 min and the pellet was suspended in 10 mM mannitol, 2 mM Tris-HCl (pH 7.4) and rehomogenized in a Polytron homogenizer. Then 1 M $CaCl_2$ solution was added to a final concentration of 10 mM and the suspension was layered over a stepwise gradient of 35%, 40% and 45% (w/v), sucrose solution containing calcium. The supernatant from the initial centrifugation was adjusted to 50% sucrose, and layered under a stepwise gradient of 30%, 40% and 45% sucrose. Tubes containing the gradients were placed in a Hitachi SRP28SA rotor and centrifuged at 141,000 xg_{max} for 90 min. Materials at each interface were collected after centrifugation. Fraction P4 was the material at the interface between the 40% and 45% sucrose layers of the gradient containing calcium; fractions G1 and G2 were the materials at the interfaces between 30% and 40%, and 40% and 45% sucrose, respectively, from the second gradient without calcium. The whole procedure was carried out at 0-4°C within 5 hours.

Immunoprecipitation and gel electrophoresis: The method decribed previously (8) was used with slight modification. The precursor and mature forms of γ-glutamyltranspeptidase were extracted by adding 0.2 volumes of a solution of 5% Triton X-100 and 100 mM sodium phosphate buffer (pH 6.0) to the subcellular fractions, and centrifuging the mixtures at 105,000 xg for 30 min at 4°C. The supernatants were mixed with excess mono-specific antibodies raised to γ-glutamyltranspeptidase, kept at 25°C for 15 min, mixed with 15-30 units of unlabeled purified γ-glutamyltranspeptidase as a carrier, and incubated further at 25°C for 45 min. The immunoprecipitate was collected by centrifugation at 3,000 rpm for 10min, washed 3 times with 2 ml of washing buffer, and subjected to SDS-polyacrylamide gel electrophoresis by the method of Baumann and Chrambach (12). After electrophoresis, gels were cut into 1 mm wide slices and dissolved in 2% sodium periodate and then their radioactivity was counted.

RESULTS AND DISCUSSION

Isolation of organelles: From our previous finding of incorporation of [^{3}H]fucose into a precursor of γ-glutamyltranspeptidase (6), precursor seems to be present in *trans* Golgi elements. To determine the distribution of the enzyme after incorporation of [^{3}H]fucose, we developed a method for isolation within 5 hours of *trans* Golgi

elements, basolateral membranes, and brush border membranes from the kidneys of two rats. UDP-galactose: ovomucoid galactosyltransferase was measured as a marker of *trans* Golgi elements, total and ouabain-sensitive Na^+, K^+-ATPases were measured as markers of basolateral membranes, and γ-glutamyltranspeptidase was measured as a marker of the brush border membranes. The activities of the marker enzymes are shown in Table 1. The contaminations of fractions G1, G2 and P4 with endoplasmic reticulum and lysosomes were low, as reported previously (8). Fraction G1 was enriched 108-fold in galactosyltransferase but had little γ-glutamyltranspeptidase or total Na^+, K^+-ATPase activity, showing that the *trans* Golgi elements in this fraction were highly purified. Fraction G2 contained total Na^+, K^+-ATPase activity, enriched 6 fold and ouabain-sensitive Na^+, K^+-ATPase. However, although G2 seemd to

Table 1. Distributions of marker enzymes in subcellular fractions of rat kidney

Markers \ Fractions		Whole homogenate	G1	G2	P4
Protein (mg)		349	0.69	1.55	1.41
γ-Glutamyl transpeptidase	S.A.* (U/mg)	1.38	1.12	1.68	15.5
	enrichment	1	0.81	1.22	11.2
Total Na^+, K^+-ATPase	S.A.* (mU/mg)	3.0	N.D.**	17.8	7.4
	enrichment	1	–	5.9	2.5
Ouabain sensitive Na^+,K^+-ATPase	S.A.* (mU/mg)	N.D.**	N.D.**	3.0	N.D.**
Ovomucoid Galactosyltransferase	S.A.* (mU/mg)	0.13	14.0	2.58	N.D.**
	enrichment	1	108	20	–

Enzyme activities in fractions G1, G2 and P4 and the whole homogenate were assayed as described in MATERIALS AND METHODS. *S.A., specific activity; **N.D., not detectable

contain the basolateral membranes, it was probably contaminated with *trans* Golgi elements because it had high galactosyltransferase activity. This fact and the suggestion that galactosyltransferase is present in basolateral membranes (13), indicated that fraction G2 was composed of basolateral membranes with small amounts of *trans* Golgi elements. Fraction P4 consisted of highly purified brush border membranes with little contamination by other organelles, because γ-glutamyltranspeptidase was highly enriched in this fraction with no detectable galactosyltransferase, ouabain-sensitive Na^+, K^+-ATPase. The relative purities of the preparations of *trans* Golgi elements and brush border membranes compared favorably with reports in the literature (14,15). Methods are available for subcellular fractionation of the epithelium of the small intestines (16), and the liver (17), but not of the kidney. Our method is convenient for isolating organelles from rat kidney with little cross-contamination.

Flow kinetics of γ-glutamyltranspeptidase: The radioactivities of proteins in fractions G1, G2 and P4 precipitated with antibody raised against γ-glutamyltranspeptidase are shown in Figure 1.

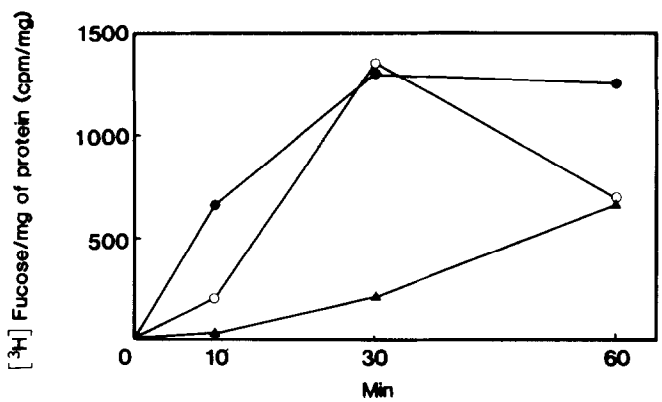

Figure 1. Incorporation of [^{3}H]fucose into the precursor and subunits in each fraction. Rats were labeled by [^{3}H]fucose for 10, 30 or 60 min, and then subcellular fractions were obtained. The radioactivity incorporated into proteins precipitated with anti γ-glutamyltranspeptidase divided by the amount of protein in each fraction was determined. Closed circles, fraction G1; open circles, fraction G2; closed triangles, fraction P4.

After injection of [^{3}H]fucose, incorporation of radioactivity into fraction G1 was significant after 10 min and increased gradually until 30 min. On the other hand, only a small amount of radioactivity was found in fraction G2 after 10 min but the radioactivity increased rapidly during the next 20 min. The radioactivity incorporated into the enzyme in fraction P4 remained low for the first 30 min but significant radioactivity was observed 60 min after the injection. Thus γ-glutamyltranspeptidase labeled with [^{3}H]fucose seems to migrate *in vivo* from fraction G1 via fraction G2 to fraction P4. Taking the data of marker enzymes (Table 1) into consideration, the radioactivity in fraction G2 is thought to be derived from the basolateral membranes. Thus some of the enzyme is probably transferred from *trans* Golgi elements to basolateral membranes and then to brush border membranes.

Determination of the site of proteolytic processing of γ-glutamyltranspeptidase: To identify the site of proteolytic processing of the precursor, we measured the relative amounts of radioactivity incorporated into the precursor and two subunits in fraction G1, G2 and P4. As shown in Figure 2, all the samples obtained were separated into three fractions with moleculer weight of 78,000, 50,000 and 23,000 daltons which were identified as those of the precursor, heavy subunit and light subunit of the enzyme, respectively. Of the total radioactivity incorporated into the enzyme, about 70% was found in precursors in fractions G1 and G2 at 10 min after the injection of [^{3}H]fucose (data not shown). After 30 min, about 80% of the total radioactivity in fractions G1 and G2 was also found as the precursor with 20% in the subunits (Figs 2a and 2b). After 60 min, 74% of the total radioactivity incorporated into the enzyme in fraction G1 and 70% of that in fraction G2 was found in precursors (data not shown). In fractions G1 and G2, the ratio of radioactivity in subunits to that

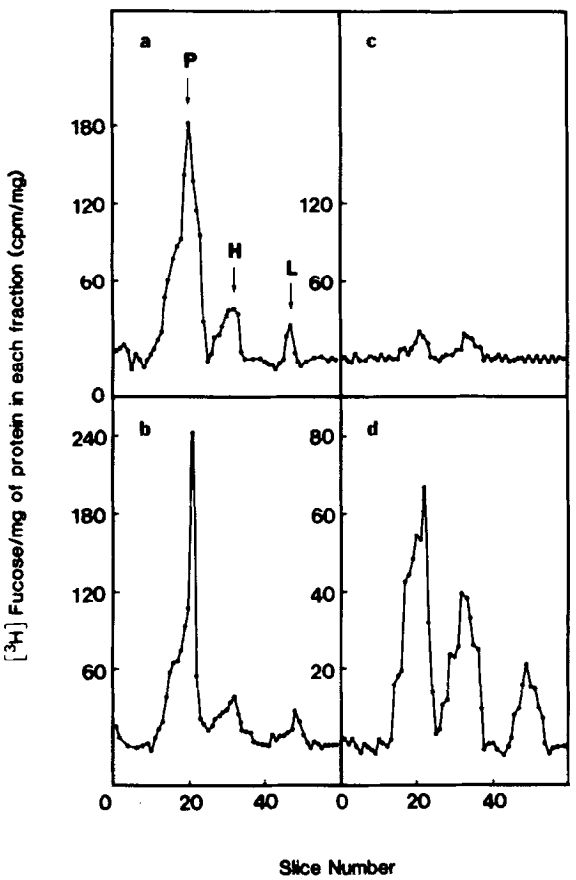

Figure 2. SDS-polyacrylamide gel electrophoresis of the immunoprecipitate of each fraction. Subcellular fractionation and immunoprecipitation were carried out as described in MATERIALS AND METHODS. a: fraction G1, 30 min after the injection; b: fraction G2, 30 min after the injection; c: fraction P4, 30 min after the injection; d: fraction P4, 60 min after the injection. P: the position of the precursor; H: the position of the heavy subunit; L: the position of the light subunit.

in whole enzyme labeled with [^{3}H]fucose was always 20-30%, irrespective of the labeling time, suggesting that these subunits are formed during the isolation of the organelles. On the other hand, fraction P4 contained little radioactivity until 30 min after the injection of [^{3}H]fucose but significant activity after 60 min as noted above (Fig. 1 and 2c). After 60 min this fraction contained 53% of the total radioactivity in the enzyme as the precursor form and 47% as subunits (Fig. 2d), whereas after 4 hours, all the radioactivity was found in the subunits (data not shown). These

results indicate that newly synthesized γ-glutamyltranspeptidase was transported to the brush border membrane as a precursor form and then converted into two subunits by protease.

Sucrase-isomaltase of the small intestine is reported to be synthesized as a precursor form with a single polypeptide chain and then converted to sucrase and isomaltase domains by exogenous protease on the membrane of microvilli (18). It would be interesting to know whether the mode of biosynthesis of γ-glutamyltranspeptidase is the same as that of other enzymes associated with the brush border membrane in the kidney and small intestine.

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REFERENCES

1. Meister, A., Tate, S.S. and Ross, L.L. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.) Vol. 3, pp.315-347, Plenum Publishing Corporation, New York, London
2. Matsuda, Y., Tsuji, A. and Katunuma, N. (1980) J. Biochem. 87, 1243-1248
3. Tsuji, A., Matsuda, Y. and Katunuma, N. (1980) J. Biochem. 87, 1567-1571
4. Matsuda, Y., Tsuji, A. and Katunuma, N. (1983) J. Biochem. 93, 1427-1433
5. Yamashita, K., Hitoi, A., Matsuda, Y., Tsuji, A., Katunuma, N. and Kobata, A. (1983) J. Biol. Chem. 258, 1098-1107
6. Matsuda, Y., Tsuji, A. and Katunuma, N. (1983) in Advances in Enzyme Regulation (Weber, G., ed.) Vol. 21, pp.103-119, Pergamon Press, Oxford, New York
7. Nash, B. and Tate, S.S. (1982) J. Biol. Chem. 257, 585-588
8. Matsuda, Y., Tsuji, A., Kuno, T. and Katunuma, N. (1983) J. Biochem., in press
9. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275
10. Fujita, M., Matsui, H., Nagano, K. and Nakao, M. (1971) Biochem. Biophys. Acta 233, 404-408
11. Bretz, R. and Stäubli, W. (1977) Eur. J. Biochem. 77, 181-192
12. Baumann, G. and Chrambach, A. (1976) Anal. Biochem. 70, 32-38
13. Weiser, M.M., Neumeier, M.M., Quaroni, A. and Kirsch, K. (1978) J. Cell. Biol. 77, 722-734
14. Fleischer, B. and Zambrano, F. (1974) J. Biol. Chem. 249, 5995-6003
15. George, S.G. and Kenny, A.J. (1973) Biochem. J. 134, 43-57
16. Ahnen, D.J., Santiago, N.A., Cezard, J.-P. and Gray, G.M. (1982) J. Biol. Chem. 257, 12129-12135
17. Fleischer, S. and Kervina, M. (1974) in Methods in Enzymology (Fleischer, S. and Packer, L., eds.) Vol. 31, pp.6-41, Academic Press, New York
18. Hauri, H.-P., Quaroni, A. and Isselbacher, K.J. (1979) Proc. Natl. Acad. Sci. USA 76, 5183-5186