Processing of the propeptide form of rat renal γ-glutamyltranspeptidase

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The biosynthesis of rat renal γ-glutamyltranspeptidase (EC 2.3.2.2) was studied by sodium dodecyl sulfate gel electrophoresis and fluorography of specific immunoprecipitates obtained at varying times' postinjection with [^{35}S]methionine. At 20 min postinjection 3 endo-β-*N*-acetylglucosaminidase H-sensitive bands were observed representing the propeptide (M_r 75 000) large subunit (M_r 49 500) and small subunit (M_r 29 000) of transpeptidase. The alterations in M_r are consistent with removal of 6 N-linked coreoligosaccharides from the propeptide; 4 from the large subunit and 2 from the small subunit. All 3 bands became more diffuse and less endoglycosidase H-sensitive by 40 min and completely resistant by 60 min postinjection. At 20 h postinjection no propeptide remained. Thus, the primary propeptide cleavage reaction occurs prior to the loss of endoglycosidase H sensitivity while about 30% of the propeptide is processed along with the heterodimer and cleaved at a later time.

γ-Glutamyltranspeptidase (EC 2.3.2.2) *Kidney proximal tubule* *Brush border membrane* *Amphipathic hydrolase* *Propeptide cleavage* *N-Linked oligosaccharide processing*

1. INTRODUCTION

Rat renal γ-glutamyltranspeptidase (EC 2.3.2.2) is localized on the external surface [1] of the brush border membrane where it functions in the interorgan turnover of glutathione [2]. The enzyme purified from detergent solubilized membranes is an amphipathic heterodimeric glycoprotein [3]. The extreme heterogeneity of the enzyme's asparagine-linked oligosaccharides [4] may be responsible for the diffuse carbohydrate- and protein-staining bands observed upon sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [3]. The small subunit (M_r 29000) of the transpeptidase contains the γ-glutamyl binding site [5,6] and is apparently associated with the brush border membrane through its interaction with the larger transmembranous [7] subunit (M_r 49500). Treatment of the amphipathic transpeptidase with papain removes the amino terminal membrane-binding domain of the large subunit [8] and produces a fully active, hydrophilic form of the enzyme [3].

Authors in [9] have identified a single-peptide precursor (M_r 78000) of the heterodimeric form of transpeptidase. This finding was established by kinetic and pulse-chase studies utilizing rat renal slices incubated in organ culture with [^{35}S]methionine or [^{3}H]fucose. The radiolabeled propeptide and subunits were immunoprecipitated and identified on fluorograms after SDS–PAGE. Since no propeptide was found in a microvillus membrane vesicle fraction 2 h after a 1 h pulse of [^{35}S]methionine, the authors concluded that cleavage of the precursor is a post-Golgi event. Due to the unknown stability of membrane structure and turnover in the organ culture system, we chose to study the biosynthesis of γ-glutamyltranspeptidase by pulse-labeling rats with an intravenous injection of [^{35}S]methionine. Our studies demonstrate that both the propeptide and the individual subunits are initially generated as

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homogeneous forms that are sensitive to treatment with endo-β-*N*-acetylglucosaminidase H (endo H). However, the endoproteolytic cleavage is incomplete and some residual uncleaved propeptide is processed along with subunits of the heterodimer to yield the heterogeneous diffuse banding forms of γ-glutamyltranspeptidase. These results indicate that cleavage of propeptide occurs primarily within the endoplasmic reticulum.

2. MATERIALS AND METHODS

White male Sprague–Dawley rats (100 g) were obtained from Zivic Miller and maintained on Purina rat chow. [^{35}S]Methionine (about 1200 Ci/mmol) and En^{3}Hance were obtained from New England Nuclear. DEAE Affi-gel blue, acrylamide and SDS-molecular mass standards were purchased from Bio Rad; aprotinin, phenylmethylsulfonyl fluoride (PMSF) benzamidine and lithium dodecyl sulfate (LDS) from Sigma; and SDS from BDH Chemicals through Gallard Schlessinger. Film for fluorography was type X-OMAT AR from Kodak. Endo H was purchased from Miles Laboratories.

2.1. *In vivo radiolabeling of γ-glutamyltranspeptidase*

Rats (100 g) were initially anesthesized by intraperitoneal injection of pentothal (0.5 mg) and then given a tail vein injection of 0.5 mCi of [^{35}S]methionine in 0.5 ml sterile saline. Rats were decapitated 20, 40, 60 min or 20 h after injection, and the kidneys were immediately excised and frozen. One kidney from each rat was demedullated, weighed and homogenized with 12 strokes of a glass teflon homogenizer in 20 volumes of 1.5 M NaCl, 2 mM benzamidine, 0.5 mM PMSF, 100 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.4. The homogenate was centrifuged at 160000 × *g* in a Ti 50 rotor for 30 min. The resulting pellet was rehomogenized with 10 vol. of 0.15 M NaCl, 2 mM benzamidine, 0.5 mM PMSF and 10 mM HEPES, pH 7.4. The resuspended membranes were adjusted to 2% Triton X-100 and centrifuged at 160000 × *g* in a Ti 50 rotor for 30 min. The resulting clear supernatant contained all of the original γ-glutamyltranspeptidase activity. The enzyme was assayed using γ-glutamyl-*p*-nitroanalide as substrate [3].

2.3. *Immunoprecipitation of γ-glutamyltranspeptidase*

γ-Glutamyltranspeptidase was purified after papain treatment of rat renal microsomes [3]. Polyclonal antibodies were raised against the pure enzyme in a goat and the IgG fraction purified by DEAE Affi-gel blue chromatography. γ-[^{35}S] Glutamyltranspeptidase was immunoprecipitated by incubating 4 or 8 units of Triton-solubilized transpeptidase activity overnight at 4°C with the appropriate amount of IgG. The sample was then centrifuged at 39000 × *g* for 15 min. The pellet was resuspended and washed first with 1 ml of 20% Triton X-100 and then with 1 ml 0.1% LDS. The final pellet was subjected to SDS–PAGE [10]. The gel was stained with Coomassie brilliant blue and destained before treating with En^{3}Hance. The gel was then dried and exposed to preflashed film at −70°C to allow accurate quantitation of the radiolabel [11]. Film was developed after 4–6 weeks and analyzed by densitometric tracing with an Helena R and D densitometer. Quantitation of radiolabel in individual bands was based on integration of the curves.

2.3. *Endoglycosidase H treatment of immunoprecipitates*

Immunoprecipitates were treated with endo H by modification of the method in [12]. The pellet was resuspended in 0.03 ml 1% LDS, 1 mM benzamidine, 0.5 mM PMSF, 10 mM sodium citrate (pH 5.0) and boiled for 5 min. The cooled sample was incubated overnight at 37°C with 1 μunit of endo H and then adjusted to the proper pH for SDS–PAGE. Control samples were treated identically except for the absence of endo H.

3. RESULTS

The transpeptidase immunoprecipitated from total cortical membranes 20 min after [^{35}S]methionine injection of rats (fig.1A) contains 3 discrete peptides. These correspond to the 2 nonidentical subunits of the purified enzyme (see fig.1B, Coomassie stain) and the propeptide. As shown in fig.2, densitometric tracing of the fluorogram in fig.1A emphasizes the existence of faint and more diffuse bands migrating immediately behind each of the 3 discrete peptides. At 40 min post injection, the diffuse bands become more

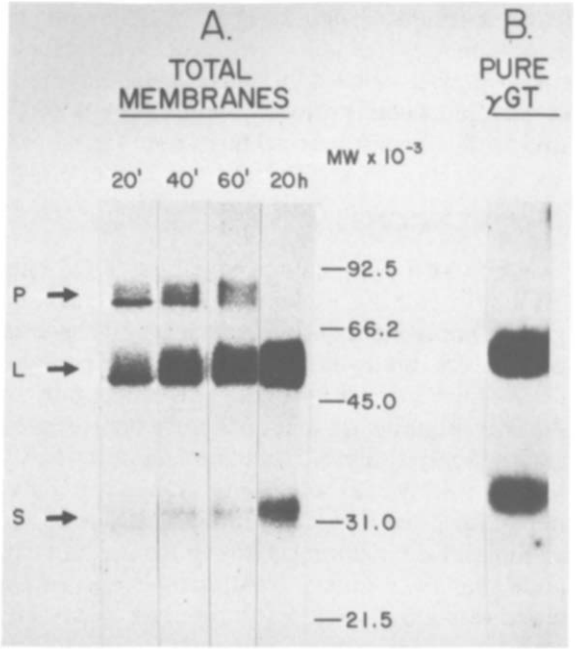

Fig.1. Identification of the propeptide form of γ-glutamyltranspeptidase. Rats (100 g) were injected with 0.5 mCi each of [^{35}S]methionine and sacrificed after either 20, 40, 60 min or 20 h, as indicated. Four units of γ-glutamyltranspeptidase were immunoprecipitated (A, left) and analyzed by fluorography after SDS–PAGE. Newly-synthesized enzyme contained the propeptide form (P) and the resulting large (L) and small (S) subunits of the heterodimer form. A sample of purified γ-glutamyltranspeptidase, stained for protein with Coomassie brilliant blue, is included for comparison (B, right). Mass standards are indicated at 92.5, 66.2, 45.0, 31.0 and 21.5 kDa.

predominant and the sharp discrete band at the leading edge becomes a minor constituent which completely disappears by 60 min post injection. The initial homogeneous forms of the 3 peptides may represent newly-synthesized and core-glycosylated peptides, while the more diffuse and slower migrating forms may be due to heterogeneity introduced by processing of the oligosaccharide side chains. Integration of the peaks in fig.2 indicate that by 20 min post injection 70% of the propeptide has been cleaved to yield the two subunits. The residual 30% of the propeptide appears to be relatively stable, persisting 2 or 3 h post injection (not shown) but disappearing by 20 h post injection.

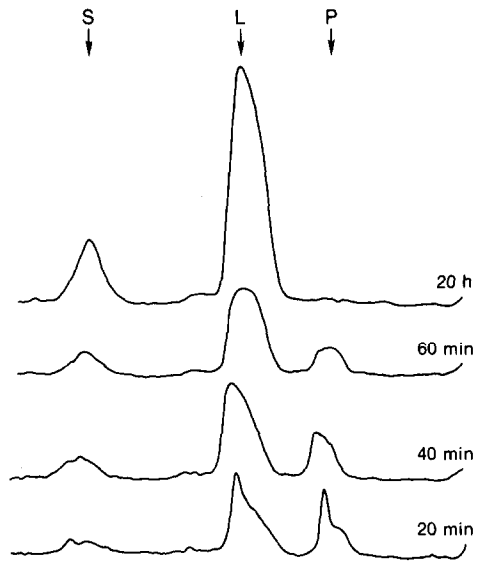

Fig.2. Analysis of the newly-synthesized forms of γ-glutamyltranspeptidase. The fluorogram in fig.1A was analyzed by densitometric tracing. Corresponding lanes are indicated by 20, 40, 60 min and 20 h. Forms of the enzyme are denoted as propeptide (P), large subunit (L) and small (S) subunit.

In order to evaluate our assumption that the oligosaccharide moieties of both the residual propeptide and heterodimeric subunits are coincidently processed, the sensitivity of the immunoprecipitated γ-glutamyltranspeptidase to endo H was determined (fig.3). At 20 min post injection, both the propeptide and the subunits contain homogeneous peptides of M_r 75000, 49500 and 29000, respectively, which are sensitive to pretreatment with endo H. This treatment produces new peptides of M_r 60000, 40000 and 23500, respectively. The alterations in M_r are consistent with identical core glycosylation of the propeptide and the heterodimer. Both exhibit a total difference in M_r of 15000. The densitometer tracings of the fluorogram (fig.4) indicate that only the faster migrating and sharp banding forms of all 3 glycopeptides are sensitive to endo H treatment. With the appearance of a more diffuse labeling pattern at 40 min post injection, less endo H-sensitive material is present and the heterogeneous forms of all 3 glycopeptides are resistant to endo H. By 60 min post injection all 3 glycopeptides exhibit diffuse banding patterns and complete resistance to endo H (not shown). This diffuse

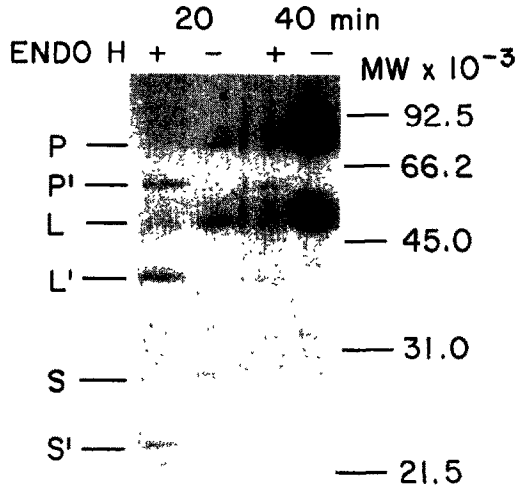

Fig.3. Endoglycosidase H sensitivity of newly-synthesized γ-glutamyltranspeptidase. Rats were injected with 0.5 mCi each of [^{35}S]methionine and sacrificed after either 20 or 40 min. Eight units of γ-glutamyltranspeptidase were immunoprecipitated in duplicate samples incubated overnight with (+) or without (−) addition of endoglycosidase H (Endo H) and analyzed by SDS–PAGE. Newly-synthesized enzyme contained the propeptide (P), large subunit (L) and small subunit (S). Peptides altered by their sensitivity to endoglycosidase H produced new bands most likely corresponding to the propeptide (P′), large (L′) and small (S′) subunits. Standards are indicated at 92.5, 66.2, 45.0, 31.0 and 21.5 kDa.

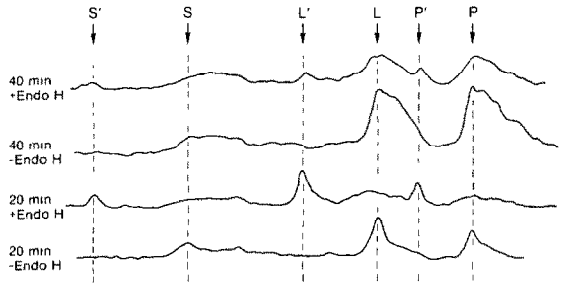

Fig.4. Analysis of the endoglycosidase H sensitivity of newly-synthesized forms of γ-glutamyltranspeptidase. The fluorogram from fig.3, with (+ Endo H) or without (− Endo H) treatment with endoglycosidase H, was analyzed by densitometric tracing. Transpeptidase propeptide (P), large subunit (L) and small subunit (S), which are sensitive to endoglycosidase H, apparently produce new bands of the propeptide (P′), large (L′) and small (S′) subunits.

banding pattern mimics the pattern of the heterodimeric subunits of the radiolabeled transpeptidase isolated 20 h post injection, as well as the Coomassie protein staining pattern of the enzyme purified from rat kidney (see fig.1).

4. DISCUSSION

At the earliest time point investigated (20 min), the majority of the propeptide is already cleaved to yield subunits. The residual uncleaved propeptide is relatively stable and disappears over a period of many hours. At 20 min both the propeptide and the two subunits are predominantly homogeneous and endoglycosidase H-sensitive, indicating the presence of species which are core glycosylated, minimally processed, and probably localized in the endoplasmic reticulum [13]. Altogether this suggests that the primary transpeptidase propeptide cleavage occurs either during or just after translation.

Authors in [4] have recently identified 157 different asparagine-linked oligosaccharides from the papain-purified rat renal γ-glutamyltranspeptidase. Utilizing hydrazinolysis and NaB^{3}H$_4$ reduction, they also report finding 4.8 mol of oligosaccharide per mol of enzyme. In addition, Frielle and Curthoys (personal communication) have identified a glycosylated peptide which is associated with the hydrophobic domain of the transpeptidase and is removed by treatment with papain. Assuming a value of 13 monosaccharides per oligosaccharide side chain removed by endoglycosidase H treatment [14], the observed alterations in M_r are consistent with the presence of 6 asparagine-linked oligosaccharides on the propeptide, 4 on the large subunit and 2 on the small subunit. All this data conforms with the proposed amphipathic structure of the transpeptidase [15,16]. In addition, the results in [4] support the hypothesis that the diffuse banding pattern of the transpeptidase which is resistant to endo H is due to the extensive heterogeneity in the processed oligosaccharide moieties. The coincident progression of both the residual propeptide and the heterodimeric subunits from endo H sensitive (homogeneous bands) to resistant (heterogeneous bands) forms, suggest that the propeptide cleavage is not essential for normal subcellular transport or oligosaccharide processing [14].

processing [14].

The cause of the incomplete cleavage of the transpeptidase propeptide prior to oligosaccharide processing is unknown. If core glycosylation and propeptide cleavage occur co-translationally, then competition between the two coincident reactions could lead to incomplete cleavage. Since transpeptidase propeptide is not present in any significant amount in the renal brush border membrane [7], the residual propeptide containing processed oligosaccharides must be cleaved by a second endoprotease after reaching the Golgi apparatus, or after insertion into the brush border membrane. Alternatively, incomplete propeptide cleavage could be due to the existence of two closely related gene products as described for parathyroid secretory protein [17]. Differences in peptide structure could alter the cleavage site within the propeptide and its susceptibility to an endoprotease of the endoplasmic reticulum. In addition, both glycosylation and oligosaccharide processing are also affected by peptide sequence. Asparagine-linked glycosylation occurs only at an Asn-X-Ser(Thr) sequence that is in the proper conformation [18]. At least one of the enzymes involved in oligosaccharide processing exhibits peptide-sequence specificity [19]. Thus, even minimal alterations in primary and secondary structures of γ-glutamyltranspeptidase could alter both the propeptide cleavage as well as the processing of oligosaccharide moieties.

The incomplete cleavage of the transpeptidase propeptide is not unique. The lysosomal enzyme, cathepsin D [20,21] and the secreted glycoprotein, the von Willebrand protein [22], contain subunit structures that result from posttranslational cleavage of a single-peptide precursor. Purified preparations of cathepsin D contain 5% of the total mass as propeptide. In the case of the von Willebrand protein, the resulting subunit and the residual uncleaved precursor are processed and secreted coincidently. However, this secreted propeptide gradually disappears with time in the medium [22].

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