The biosynthesis of γ-glutamyl transpeptidase was investigated in hepatoma tissue culture cells. Pulse-chase experiments using [35S]methionine labeling have shown that the two glycosylated subunits of the enzyme (Mr = 58,000 and 29,000) derive from a single glycosylated precursor (Mr = 79,000 at early times). Only one polypeptide chain was immunoprecipitated from cell-free translation products and was shown to correspond to the nonglycosylated precursor (Mr = 64,000). Treatment with endoglycosidase H was used to probe for the transfer of the proteins from the endoplasmic reticulum to the Golgi and demonstrated: (i) that the precursor is at least partially cleaved in the endoplasmic reticulum; (ii) that part of the precursor is transferred to the Golgi where the processing of the oligosaccharide chains takes place. None of the precursor forms were detected at the surface of the cell where the mature enzyme was found. Tunicamycin, an inhibitor of protein glycosylation, did not prevent the proteolytic processing of the enzyme, but delayed the appearance of the mature enzyme at the cell surface. Monensin, which is known to alter Golgi functions, significantly delayed the acquisition of complex type oligosaccharides and the appearance of the enzyme at the cell surface. It did not, however, alter the proteolytic processing of the precursor of γ-glutamyl transpeptidase. Taken together, these results show that γ-glutamyl transpeptidase is synthesized as a single precursor which is at least partially cleaved in the endoplasmic reticulum. Part of the precursor is transferred to the Golgi where its oligosaccharide chains are processed.

γ-Glutamyl transpeptidase (EC 2.3.2.2) is the enzyme which catalyses the hydrolysis of γ-glutamyl compounds and the transfer of the γ-glutamyl moiety of glutathione and its derivatives to certain amino acids or dipeptides (1). It is involved in glutathione metabolism and probably in amino acid uptake (2, 3). The enzyme is localized at sites involved in transport, such as the renal and intestinal brush-borders (2, 3). Although most of the studies dealing with its structure and function have been performed on the kidney enzyme, γ-glutamyl transpeptidase of liver and liver-derived cells has attracted considerable attention. Indeed, the rat liver enzyme is induced by a number of hormones such as glucocorticoids (4) and drugs such as ethanol and carcinogens (5, 6). We have recently established hepatoma cell culture systems where similar effects were reproduced and analyzed (7–9). Considerable differences in γ-glutamyl transpeptidase activity were found between the various hepatoma cells studied.

γ-Glutamyl transpeptidase is an amphipathic heterodimeric glycoprotein (2). The light subunit carries the active site and is anchored to the membrane through its interaction with the heavy subunit. Recent studies using whole animals (10, 11) and kidney slices (12) suggest that the two subunits may be synthesized as a single precursor in rat kidney; however, conflicting results were obtained as to where the proteolytic processing takes place. In addition the asparagine linked oligosaccharides have been analyzed recently and found to be extremely heterogeneous (13). It appears therefore that γ-glutamyl transpeptidase undergoes significant post-translational modifications including proteolytic processing and glycosylation. It is now accepted that protein glycosylation occurs in several steps: addition of high mannose oligosaccharides to asparagine residues takes place in the endoplasmic reticulum and is cotranslational (14). The enzymes responsible for the processing of these oligosaccharides and for the addition of galactose, fucose, and sialic acid leading to the mature protein are believed to be mainly located in the Golgi (15). The sites of proteolytic processing are not as precisely identified, except for the removal of the signal peptide of certain membrane and secreted proteins (16). In fact, the relationships between the steps of glycosylation, the proteolytic processing, and the rate of transfer to the plasma membrane are not well understood. Our study of the biosynthesis of γ-glutamyl transpeptidase had therefore focused on the analysis of the post-translational modifications of this plasma membrane protein. We have also provided evidence for the synthesis of the two subunits as a single precursor using cell-free translation experiments. In the present study, we have used the HTC cell, a rat hepatoma-derived cell line, for the following reasons: (i) this cell exhibits high γ-glutamyl transpeptidase activity as compared to other hepatoma cells; (ii) it has been used and characterized for the study of membrane and secreted protein biogenesis and turnover (17).
Biosynthesis and Processing of HTC \( \gamma \)-Glutamyl Transpeptidase

**MATERIALS AND METHODS AND RESULTS**

**DISCUSSION**

In this article, we present evidence for a common precursor of the two subunits of \( \gamma \)-glutamyl transpeptidase and we analyze the post-translational modifications of this enzyme. The existence of a common precursor is suggested by the following observations: (i) pulse-chase experiments using [\( ^{35} \)S]methionine as the radioactive label show that a \( M_r = 79,000 \) species is the first \( \gamma \)-glutamyl transpeptidase immunorelated peptide to be visualized. As this polypeptide disappears, the heavy and light subunits of the enzyme appear (Fig. 2); (ii) using tunicamycin-treated cells, we show that the various immunoprecipitated species correspond indeed to three distinct polypeptides (precursor, heavy and light subunits). All the immunoprecipitated species in control cells correspond to glycosylated forms of these polypeptides (Fig. 4B); (iii) cell-free synthesis experiments provide strong support for the precursor hypothesis. A single polypeptide species is immunoprecipitated from translation products of HTC cells RNA. This polypeptide has a similar migration on sodium dodecyl sulfate-polyacrylamide gels as the precursor peptide isolated from tunicamycin-treated cells (Fig. 5). It, therefore, corresponds to the nonglycosylated form of \( \gamma \)-glutamyl transpeptidase precursor. We cannot conclude from our data if there is a cleavage of the signal peptide of the enzyme. However, the nonglycosylation of signal peptides has been reported for several other \( M_r \), terminally-sulfated proteins (27–29). Furthermore, recent results demonstrated that there is no cleavage of the signal peptide of \( \gamma \)-glutamyl transpeptidase in rat kidney (30–32).

Our study has focused on the post-translational modifications of the precursor of \( \gamma \)-glutamyl transpeptidase and its subunits. The proteolytic processing and the glycosylation steps have been analyzed using endoglycosidase H sensitivity, \([\text{H}]\)fucose and \([\text{S}]\)methionine labeling, as well as the effect of tunicamycin and monensin. The following observations suggest that the proteolytic processing occurs in the endoplasmic reticulum: (i) at early chase times, both the precursor and the subunits are found in an endoglycosidase H sensitive form (Fig. 6); (ii) in monensin-treated cells, the processing of the precursor to its subunits is not inhibited whereas the processing of the oligosaccharide chains into complex type oligosaccharides is significantly delayed (Fig. 8). These results mean that at least part of the precursor pool is processed before being transferred to the Golgi complex where many enzymes responsible for the maturation of the oligosaccharide chains are located (15).

Our data also suggest that part of the uncleaved precursor is transferred to the Golgi complex: (i) high molecular weight forms of the precursor (\( M_r = 88,000 \)) are endoglycosidase H-resistant (Fig. 6); (ii) these forms are labeled with \([\text{H}]\)fucose as the subunits are; (iii) they do not return to monensin-treated cells (Fig. 4A). Since no precursor forms are detected at the surface of HTC cells (Fig. 7), then, the \( M_r = 88,000 \) precursor form is probably located in the Golgi complex. Taken together, all these results allow us to draw the following scheme for the biosynthetic pathway of \( \gamma \)-glutamyl transpeptidase: the protein is synthesized as a single precursor peptide which acquires cotranslationally high mannose core glycosylation. Our data do not provide evidence for the removal of a signal peptide at this stage. During its journey in the endoplasmic reticulum the precursor is cleaved, yielding the two subunits of the enzyme. The processed enzyme is transferred to the Golgi where it acquires complex type oligosaccharides and then attains the plasma membrane. However, part of the precursor is not cut in the endoplasmic reticulum and undergoes the processing of its oligosaccharides in the Golgi complex. Since it is not detected at the surface, it might be cut in the Golgi to yield the two subunits. Alternatively, it could attain the plasma membrane and be very rapidly processed to its subunits.

The biosynthesis of \( \gamma \)-glutamyl transpeptidase in whole rat kidney or in kidney slices has been studied recently (11–13). Conflicting results were obtained as to where the proteolytic processing occurs. Authors have argued that, since the precursor was labeled with \([\text{H}]\)fucose, it was probably cleaved in the Golgi (13) or in post-Golgi membranes (22), which is in some recent studies. In view of our data, such results are not unexpected since \([\text{H}]\)fucose would only label the fraction of the precursor which has not been cleaved in the endoplasmic reticulum and which has therefore undergone the complex glycosylation in the Golgi; the precursor that is found and cut in the endoplasmic reticulum is not visualized by \([\text{H}]\)fucose labeling. Capraro and Hughey (10) have suggested that the precursor might be cleaved in the endoplasmic reticulum in rat kidney using in vivo \([\text{S}]\)methionine labeling and endoglycosidase H sensitivity. These authors have also suggested that part of the uncleaved precursor might be transferred to the Golgi complex. This in vivo observation is in good agreement with our data and suggests that our scheme for the biosynthesis of \( \gamma \)-glutamyl transpeptidase in HTC cells applies to other cells and tissues.

The biosynthesis of multimeric secretory and membrane-bound proteins has been investigated recently in various systems. Different mechanisms have been found. The subunits of the acetylcholine receptor (33), HLA A and HLA B antigens (34), IgM (35), 36), are not synthesized as a single precursor. In these cases, the subunits assemble post-translationally, thus triggering the maturation of the whole protein (34, 36). In many other cases, a single precursor is first synthesized and is then cleaved post-translationally. However, the site of cleavage varies considerably. Certain proteins such as sucrase isomaltase (37, 38), haptoglobin (39, 40), and apolipoprotein A-1 (41) are cleaved by extracellular proteases after they reach the cell surface or after they are secreted in the serum. Other proteins as well as propolypeptide hormones are known to be processed either in the Golgi or in secretory granules (42, 43). Finally, some proteins are cleaved in the endoplasmic reticulum (39, 44). In a few cases, the site of cleavage is not restricted to one compartment; haptoglobin is cleaved in the endoplasmic reticulum as well as in the serum (39); the insulin receptor precursor is probably cleaved in the Golgi since monensin inhibits its processing, however, immature subunits are detected at early times (43). In this study we have shown that although most of the precursor of \( \gamma \)-glutamyl transpeptidase is cleaved before its maturation in the Golgi, part of it is found uncleaved in an endoglycosidase H-resistant form. It therefore appears that whereas the sites of the glycosylation steps seem to be similarly localized for all glycoproteins, the sites of proteolytic processing of plasma membrane or secreted proteins are not. One possible expla-
nation for this could be that each compartment contains proteases but that the specificities of these proteases vary from one compartment to another. Comparing the sequences of the cleaved proteins should provide a test for this hypothesis. Very recently, several groups (44-46) have demonstrated that proteins are transferred from the endoplasmic reticulum to the Golgi at characteristic rates but that these rates differ considerably from one protein to the other. Therefore, there is a certain degree of specificity in the post-translational modifications of plasma membrane and secreted proteins at least for transfer rates and proteolytic processing. Further investigations are needed to determine how this specificity is achieved.

A major area of investigation in protein processing is the search for "signals" that trigger the various steps of maturation. Signals could include part of the peptide chain such as leader peptide or of the sugar moiety such as mannose-6-phosphate which is specific for lysosomal enzymes (16). Glycosylation is not required for the proteolytic processing of γ-glutamyl transpeptidase but that the specificities of these proteases vary considerably from one protein to the other. Therefore, there is a certain degree of specificity in the post-translational modifications of plasma membrane and secreted proteins at least for transfer rates and proteolytic processing. Further investigations are needed to determine how this specificity is achieved.

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Biosynthesis and Processing of HTC γ-Glutamyl Transpeptidase

Supplementary material to
Biosynthesis and Processing of HTC γ-Glutamyl Transpeptidase

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Material and Methods

Biosynthesis and Processing of HTC γ-Glutamyl Transpeptidase was performed in a 50 ml shake flasks containing 15 ml of broth (300 ml/litre peptone, 150 ml/litre soybean meal, 150 ml/litre tryptone, 150 ml/litre yeast extract, and 150 ml/litre glucose). After 48 hours, the broth was harvested, centrifuged at 10,000 x g for 30 minutes, and the supernatant was discarded. The pellet was washed with 0.1 M phosphate buffer, pH 7.0, and then resuspended in 10 ml of 0.1 M phosphate buffer, pH 7.0. The suspension was then filtered through a 0.45 μm filter and the filtrate was used for further analysis.

Results

Figure 1: SDS-polyacrylamide gel electrophoresis of HTC γ-Glutamyl Transpeptidase. The enzyme was separated by SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue R-250. The bands were visualized by UV light. The migration of the bands was as follows: band 1 (14 kDa), band 2 (20 kDa), band 3 (24 kDa), and band 4 (30 kDa).

Figure 2: Pulse-chase labeling of HTC γ-Glutamyl Transpeptidase with [35S]methionine. Cells were grown in the presence of [35S]methionine and the chase was performed with cold methionine at various time points. The results were expressed as counts per minute per milligram of protein.

Figure 3: Labeling of HTC γ-Glutamyl Transpeptidase with [35S]methionine. Cells were grown in the presence of [35S]methionine and the chase was performed with cold methionine at various time points. The results were expressed as counts per minute per milligram of protein.

Figure 4: Time course of HTC γ-Glutamyl Transpeptidase activity. The enzyme activity was measured by the release of γ-glutamyl cysteine from γ-glutamyl cysteine-ligand in the presence of γ-glutamylcysteine-ligand. The enzyme activity was expressed as units per milligram of protein.

Figure 5: Effect of temperature on HTC γ-Glutamyl Transpeptidase activity. The enzyme activity was measured at various temperatures ranging from 10°C to 50°C. The results were expressed as units per milligram of protein.

Figure 6: Effect of pH on HTC γ-Glutamyl Transpeptidase activity. The enzyme activity was measured at various pH values ranging from 6.0 to 10.0. The results were expressed as units per milligram of protein.

Figure 7: Effect of metal ions on HTC γ-Glutamyl Transpeptidase activity. The enzyme activity was measured in the presence of various metal ions at a concentration of 1 mM. The results were expressed as units per milligram of protein.
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\text{biosynthesis and processing of HTC } \gamma-\text{glutamyl transpeptidase}
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In a glycosylation study, we labeled HTC cells with [3H]fucose for various periods of time. It is seen well that fucose is added to glycoproteins in the Golgi membranes. After a pulse of 20 min, we did not detect any labeled species, suggesting that the Mr 79,000 species does not contain fucose residues (Figure 1). After a labeling time of 1 or 3 hours, both subunits were detected and faint bands corresponding to high molecular weight precursor forms were visualized (approximately 85,000).

To determine the role of glycolysis in the appearance of the various bands, we performed [3H]methionine labeling experiments in the presence of tunicamycin, a potent inhibitor of protein glycosylation. After a chase of five hours, only three main bands (Mr 85,000, Mr 55,000, and Mr 200,000) were observed in cells treated with tunicamycin for 4 hours (Figure 4B). When HTC cells were treated for 1 hour with the drug, only one of these three species was detected (data not shown). This treatment is compatible with the appearance of the precursor, heavy and light subunits of 

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\text{gamma-glutamyl transpeptidase. Therefore, the numerous diffuse bands observed in the absence of tunicamycin correspond to glycoprotein forms of these three polypeptides.}
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Further substantiation that 

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\text{gamma-glutamyl transpeptidase subunits are synthesized as a single precursor, we isolated PAM from HTC cells and studied the products of cell-free translation. Figure 5 shows that a single polypeptide is specifically immunoprecipitated under these conditions. The molecular weight of this polypeptide is 80,000, very similar to the molecular weight of the precursor peptide detected in tunicamycin-treated cells. This observation}
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\text{provides evidence that the precursor is a homogeneous polypeptide, and}
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\text{that the full processing of the precursor to the mature enzyme is taking place in vivo.}
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We used endoplasmic reticulum as an analytical tool to probe for the transfer of 

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\text{gamma-glutamyl transpeptidase from the rough endoplasmic reticulum to}
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\text{the Golgi complex. Polypeptides with high mannose type oligosaccharides added in the endoplasmic reticulum are sensitive to}
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\text{endo-glucosidase in treatment with complex type oligosaccharides found in the Golgi}
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\text{membranes are not. After a 30 min pulse, the glycoprotein precursor is sensitive to}
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\text{endo-glucosidase I treatment (Figure 6A). Following a 10 min chase, the precursor as well as the}
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\text{sharp bands corresponding to the subunits were endoglycosidase H sensitive. The diffus}
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\text{e bands of the precursor and the subunits which appear at 80 min were resistant to this enzyme}
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\text{treatment; these glycosylated polypeptides were still resistant after a chase of 180 min. It is}
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\text{not clear} \quad \text{REPEATED}
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\text{whether these bands corresponded to endoglycosidase H sensitive polypeptides (Figure 6B).}
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\text{Tunicamycin, added 8 hours before labeling, was present during labeling time.}
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\text{Immunoprecipitation of Triton extracts was then carried out as described above.}
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We determined the effect of glycosylation in the proteolytic processing of 

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\text{gamma-glutamyl transpeptidase, we performed pulse-chase experiments in control and tunicamycin-treated}
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\text{cells (Figure 8). Figure 8 shows that endoglycosidase treatment does not prevent the conversion of the precursor into the}
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\text{two subunits of gamma-glutamyl transpeptidase. This result suggests that the oligosaccharide}
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\text{residues do not provide the signal for the proteolytic processing of the enzyme. The}
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\text{effect of tunicamycin on the transfer of the enzyme to the plasma membrane has also been evaluated. A delay in the appearance of gamma-glutamyl transpeptidase at the cell surface was observed (Figure 7). The subunits were no longer detected at the surface after a one hour chase but after a three hour chase, the two subunits were again observed. The delay in the appearance of the enzyme is only minimal. Moreover, the estimated molecular weight of the heavy and light subunits are 90,000 and 68,000 respectively in monensin treated cells. It also appears that these bands are not as diffuse as in control cells. It is noticeable that the proteolytic processing of the precursor takes place in the presence of monensin suggesting that the proteolytic cut occurs in a compartment not altered by this drug.}
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The effect of 

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\text{endoglycosidase H on gamma-glutamyl transpeptidase-related post-Golgi}
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\text{membrane treated cells was determined. After a one hour chase the precursor and the two sub}
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\text{units were no longer detected at the surface. Incubation of PAM with endoglycosidase}
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\text{H for 30 min in the presence of 100 mg/ml of}
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\text{endoglycosidase H in control cells (Figure 8). At three hour chase, the heavy and light subunits were still sensitive to this enzyme treatment but to a lesser extent (Figure 8). Therefore monensin delays the processing of high mannose oligosaccharides chains but does not prevent the appearance of a partial resistance to endoglycosidase H.}
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Since monensin is known to alter the intracellular transport of plasma membrane proteins (29), we examined the appearance of gamma-glutamyl transpeptidase at the cell surface of monensin-treated cells. Figure 7 shows that the heavy and light subunits are still detected at the cell surface after a one hour chase. The appearance of gamma-glutamyl transpeptidase at the cell surface is striking that the appearance of the enzyme at the cell surface is accompanied by the acquisition of a partial resistance to endoglycosidase H.

Finally we determined the effect of tunicamycin and monensin on gamma-glutamyl transpeptidase activity. Both drugs do not alter the enzyme activity even after an incubation time of 24 hours (data not shown).