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Glycosylation of human chorionic gonadotropin in mRNA-dependent cell-free extracts: Post-translational processing of an asparagine-linked mannose-rich oligosaccharide

(carbohydrate processing *in vitro*/glycoprotein hormone/cell-free protein synthesis)

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ABSTRACT **Placental RNA has previously been shown to direct the synthesis of an asparagine-linked mannose-rich glycosylated form of the α subunit of human chorionic gonadotropin (hCG-α) in lysates derived from mouse ascites tumor cells. Glycosylation was dependent on the presence of homologous microsomal membranes, and the glycosylated protein was sequestered into the microsomal vesicles. Here we show that when Triton X-100 is added after 60 min of translation and the incubation is continued, there is a shift of this glycosylated form to new discrete lower molecular weight proteins. The formation of these new proteins was not the apparent result of proteolysis because (*i*) treatment of the fully glycosylated protein or the proteins formed in the presence of Triton with endoglycosidase H resulted in the formation of a single protein migrating at the same rate on sodium dodecyl sulfate gels; (*ii*) the migration of nonglycosylated hCG-α synthesized in the presence of membranes isolated from tunicamycin-pretreated ascites tumor cells was not changed upon Triton addition; and (*iii*) the Triton-induced change was inhibited by mannonolactone, yeast mannan, and purified mannose oligosaccharides. It was also shown that little processing of the mannose-rich glycoprotein occurred in the presence of microsomal membranes alone. However, addition of the ribosome-free supernatant fraction to the glycoprotein resulted in processing. These data suggest that processing of the oligosaccharide core is a compartmentalized process in which removal of sugar, presumably mannose, requires a transfer of the glycoprotein from the endoplasmic reticulum to another component of the secretory cascade.**

One intriguing aspect concerning the expression of many eukaryotic secretory proteins is the extensive protein modifications that occur during synthesis. For example, the prepeptide, located at the amino terminus of nascent polypeptide chains synthesized on membrane-bound polysomes, can be excised prior to the release of the completed protein (1, 2). The attachment of an oligosaccharide core to specific asparagine residues in glycoproteins also occurs on nascent chains (3–6). Thus, unfinished protein chains serve as substrates for membrane-dependent reactions that are pivotal steps for the processing and transport of a variety of secretory proteins.

Recently, it has been observed that the oligosaccharide core that is transferred to specific asparagine residues via a dolichol intermediate contains more mannose residues than are found in the mature glycoprotein form (7–10). Current evidence strongly suggests that the structure of the asparagine-linked nascent core is very similar for several glycoproteins (7–9). Studies dealing with sugar composition have shown that the maturation of the mannose-rich nascent core attached to the G protein of vesicular stomatitis virus proceeds through stepwise removal of carbohydrate (7–10). Thus, one or more trimming reactions are apparently obligatory before the further coupling of sugars unique to specific glycoproteins can occur.

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Previously we reported that, in membrane-supplemented cell-free extracts derived from ascites tumor cells, placental mRNA directed the synthesis of a glycosylated form of the α subunit of human chorionic gonadotropin (hCG-α) containing a mannose-rich oligosaccharide unit (6). In addition, intermediate glycosylated forms with apparent molecular weights lower than the molecular weight of the initial glycosylated protein were observed. Here we present evidence suggesting that these intermediates contain less carbohydrate than the fully glycosylated form. The data suggest that the mannose-rich glycoprotein was processed *in vitro*.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine was obtained from Amersham. Concanavalin A coupled to agarose, methyl α-D-mannopyranoside, and yeast mannan were purchased from Sigma. D-Mannono-1,5-lactone and endoglycosidase H were obtained from P-L Biochemicals and Miles, respectively. Tunicamycin was a gift from G. Tamura of the University of Tokyo, Tokyo, Japan. Mannose tri- and tetrasaccharides were a generous gift of Clinton Ballou. hCG-α antiserum was prepared by S. Berkin and R. Canfield as described in ref. 11.

General Methods. The preparation of cell sap (S-100), ribosomes, and membranes derived from mouse ascites tumor cells and the isolation of human placental mRNA have been described (1). Protein synthesis was assayed in 60-μl reaction mixtures containing 0.1 μM [^{35}S]methionine according to Szczesna and Boime (1). Immunoprecipitation of the cell-free products and their resolution on sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gels were carried out as described (6).

Isolation of Glycosylated Product. Scaled up reaction mixtures (1.2 ml) containing membranes and lysate derived from ascites tumor cells and placental mRNA were incubated at 31°C for 60 min, and then an equal volume of a buffer containing 120 mM KCl, 30 mM Tris (pH 7.5), 5 mM magnesium acetate, and 7 mM 2-mercaptoethanol (buffer A) was added. The samples were then centrifuged at 120,000 × *g* for 3 hr. The supernatant was discarded and the pellets (containing ribosomes and membranes) were washed three times with buffer A. This procedure permitted us to isolate the glycosylated α subunit associated with membrane vesicles (Fig. 1, band D). The pellets were then resuspended in 300 μl of buffer A or in the amount of S-100 that was equal to that present in the original reaction mixture. These reconstituted samples were incubated for 90 min at 31°C, treated with Triton X-100 and deoxycholate, centrifuged, and immunoprecipitated with α subunit-specific antisera as described (6). The glycosylated

Abbreviations: hCG, human chorionic gonadotropin; hCG-α, α subunit of hCG; $NaDodSO_4$, sodium dodecyl sulfate.

products were treated with endoglycosidase H according to Bielinska and Boime (6).

Preincubation of Ascites Tumor Cells with Tunicamycin. Ascites tumor cells were harvested from mice and washed with phosphate-buffered saline to remove erythrocytes according to Aviv *et al.* (12). The cells were diluted to a final concentration of 10^7 per ml with Eagle's minimal essential medium (13) containing tunicamycin at 1 μg/ml. This suspension was placed in spinner bottles, gassed with 95% CO_2/5% O_2 and incubated at 37°C for 4 hr. The cells were washed three times with Tris-buffered saline (12) and lysed, and the membranes were obtained as discussed above (1).

RESULTS

Previously we showed that first trimester placenta mRNA, in a reconstituted ascites tumor cell-free system containing microsomal membranes, directed the synthesis of a glycosylated form of the α subunit of hCG (6). This protein was bound specifically to a concanavalin A affinity column, and its migration on $NaDodSO_4$ gels was enhanced by the action of α mannosidase or endoglycosidase C-2 or H (6). The action of endoglycosidases requires an oligosaccharide containing di-*N*-acetylchitobiose and several mannose residues. On the basis of the sensitivity of band D to these glycosidases and the observations that the dolichol unit contains more mannose residues than are found in the mature hormone form, we presumed that band D contained a nascent core enriched for mannose.

During these studies we used Triton X-100 to interrupt further membrane action and permit examination of the kinetics of glycosylation of the α subunit (6). Depending on the time of detergent addition, a series of intermediate migrating bands was observed on the gels. When added at the start of the incubation, Triton inhibited the formation of the fully glycosylated form (band D, Fig. 1), but another intermediate migrating faster than band D was observed (band B). However, if Triton was added with cycloheximide (to inhibit additional protein synthesis) 60 min after the initiation of protein synthesis and the incubation was continued, there was an apparent time-dependent shift of band D to new discrete proteins (bands M and A). At increasingly longer times of incubation after Triton addition, little, if any, band D and more protein migrating in the region of band A (band A′) was observed. These data suggested that Triton treatment exposed band D to the action of either a glycosidase or a protease. To investigate this point, the products synthesized in the presence of Triton were treated with endoglycosidase H. This enzyme splits the asparagine-bound di-*N*-acetylchitobiose, leaving behind a terminal asparaginyl-linked *N*-acetylglucosamine. Treatment of band D with this enzyme causes a shift in the migration of this glycosylated protein (6). If band M was the result of proteolytic cleavage, endoglycosidase treatment should result in a protein migrating faster than the corresponding product formed from band D in the presence of the enzyme. When endoglycosidase H was added to a mixture containing bands D and M, or band D alone, it was observed that in both cases the resulting products from the enzyme treatment migrated at the same rate (Fig. 2). These data suggested that at least band M was not derived from band D by proteolysis, and moreover that band M contained a sufficient number of mannose residues to render it susceptible to the action of endoglycosidase H.

To further show that the Triton-induced alteration in the migration of the protein was due to a change in the carbohydrate content in the protein, membranes isolated from tunicamycin-treated ascites tumor cells were used. Tunicamycin prevents the formation of *N*-acetylglucosamine-lipid intermediates that serve as donors for the synthesis of mannose-rich oligosaccharide units (14–16). When membranes derived from cells treated with tunicamycin were added to the cell-free system, band D was not observed, but rather a protein migrating much faster (Fig. 3). The migration of the faster protein was not sensitive to the action of endoglycosidase H or of α-mannosidase (data not shown). Thus, tunicamycin inhibited the synthesis of most, if not all, of the oligosaccharide component of the lipid-linked sugar intermediates. Therefore, we would expect that if carbohydrate was removed in the presence of Triton, the migration of the tunicamycin protein would be in-

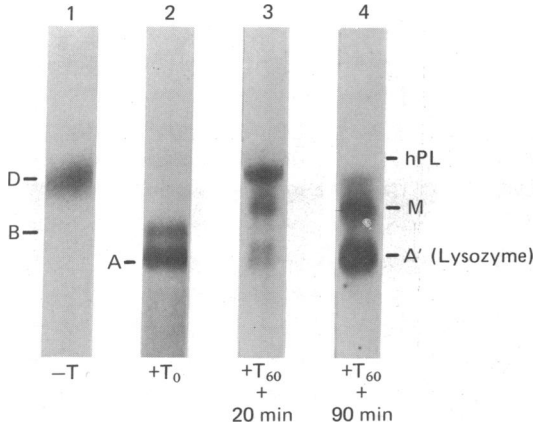

FIG. 1. Fluorograph of a $NaDodSO_4$ polyacrylamide gel electropherogram of [^{35}S]methionine-labeled protein isolated by immunoprecipitation. Reconstituted S-100/ribosome extracts derived from ascites tumor cells were incubated in the presence of ascites tumor membranes. Lane 1, no Triton X-100 (T). Lane 2 corresponds to the addition of Triton (0.04% final concentration) at the start of a 90-min incubation period. Lanes 3 and 4 indicate reaction mixtures that were incubated in the absence of Triton for 60 min. Triton was then added and the incubation was continued for 20 or 90 min. Band D denotes the migration of the initial glycosylated form of hCG-α. Band A refers to the nonglycosylated pre-form of hCG-α, whereas A′ denotes the protein band whose formation is Triton dependent and that comigrates with band A and the lysozyme marker (M_r 14,500). hPL, Human placental lactogen marker (M_r 22,200).

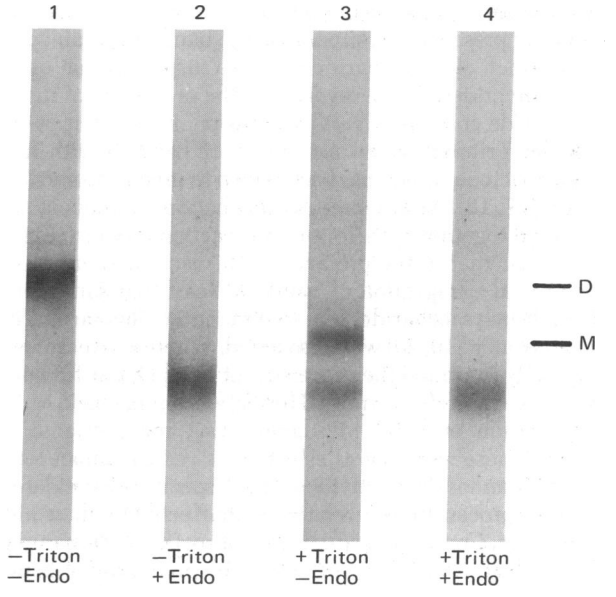

FIG. 2. Effects of endoglycosidase H (Endo) on proteins synthesized in membrane-supplemented ascites tumor lysates in the presence and absence of 0.04% Triton X-100. In lanes 3 and 4, Triton was added to the reaction mixtures after 60 min of incubation, and the incubation was continued for an additional 90 min.

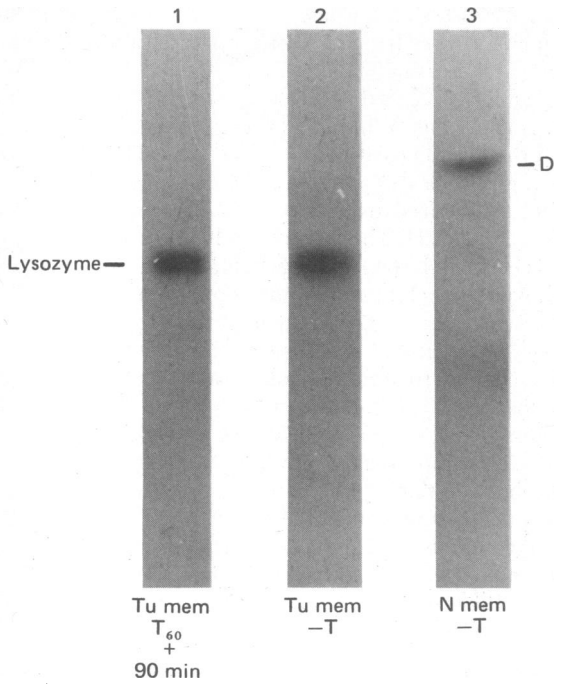

FIG. 3. The distribution of protein synthesized in the presence of comparable amounts of membranes prepared from control (N mem) or tunicamycin-treated cells (Tu mem). Where indicated, Triton (T) was added after 60 min of incubation and the reaction mixture was incubated for an additional 90 min.

sensitive to Triton addition. Accordingly, Triton was added to cell-free extracts containing tunicamycin-treated membranes after 60 min and the incubation was continued for an additional 90 min (Fig. 3). It is clear that the migration of the protein did not change in the presence of Triton. These further data suggest that the enhanced migration of protein resulting from the post-translational addition of Triton to lysates containing normal membranes was due to a modification of the carbohydrate component present in band D.

It was clear that two major intermediate bands were generated in the presence of Triton. Because band D contained the mannose-rich oligosaccharide core, we suspected that one or more mannosidases were involved in the processing of the oligosaccharide core. To investigate this point we attempted to block the Triton-induced processing of band D with mannono-1,5-lactone, which has been shown to inhibit mannosidase activity (17, 18). After 60 min of incubation mannonolactone was added together with Triton to a reaction mixture (Fig. 4). It was clear that in the presence of this lactone there was no change in the migration of band D. Yeast mannan, a mannose-rich polysaccharide, and two mannose oligosaccharides derived from it (19, 20) were also tested. Whereas yeast mannan completely inhibited the processing of band D, purified mannose oligosaccharides permitted formation of some band M. The accumulation of band M suggests that mannotriaose and mannotetraose were not as effective as yeast mannan for inhibiting mannosidase activity or that a specific glycosidase involved in processing was relatively unaffected by these oligosaccharides. Comparable quantities of methyl α-D-mannopyranoside had little, if any, effect on the processing of band D (data not shown).

It appeared from the above data that band D was exposed to one or more mannosidase activities in the cell-free system. To examine whether an activity in the membrane, in the S-100 fraction, or in both was processing band D, membranes con-

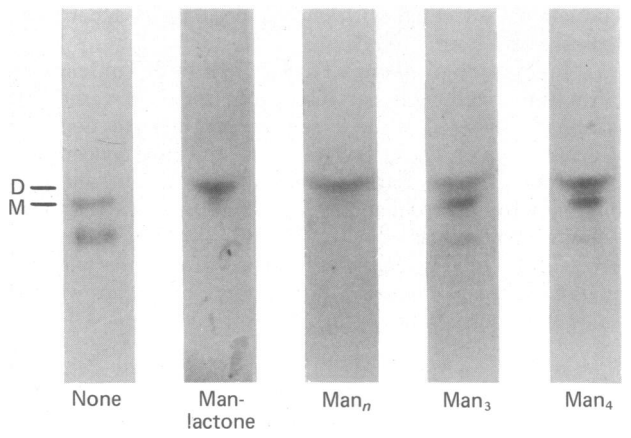

FIG. 4. Effects of yeast mannan (Man_n, 5 mg/ml), mannonolactone (Man-lactone, 100 mM), mannotriaose (Man_3, 30 mg/ml), and mannotetraose (Man_4, 30 mg/ml) on the distribution of proteins synthesized in the presence of 0.04% Triton X-100. After 90 min of incubation the carbohydrates were added together with Triton. The concentrations expressed are final concentrations. The reaction mixtures were immunoprecipitated as described.

taining the primary glycosylated hCG-α were collected from scaled-up reaction mixtures as described in *Materials and Methods*. It was shown from previous experiments that band D was sequestered into microsomal membranes (21). Membranes containing glycosylated hCG-α were resuspended in buffer A or in the presence of S-100 and the reactions were incubated for 90 min at 31°C (Fig. 5). Without Triton addition no detectable change in migration of band D was observed in either the presence or the absence of S-100. However, the addition of 0.5% Triton resulted in processing of band D only when the membrane pellet was resuspended in the presence of the S-100 fraction. Thus, the membrane fraction alone was insufficient for processing a significant amount of band D. In this case, as in previous experiments using post-translational

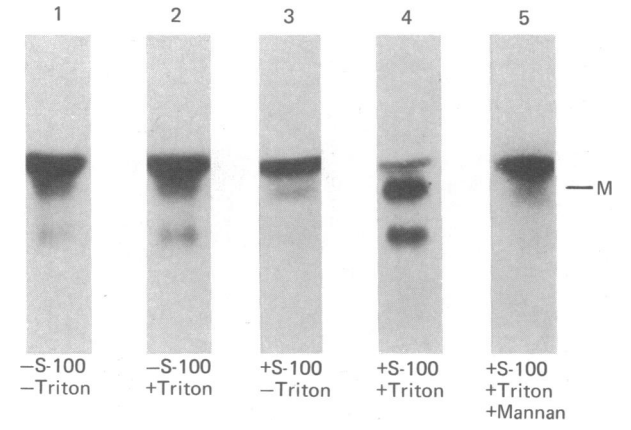

FIG. 5. Addition of ascites tumor S-100 to membrane-sequestered primary glycosylated hCG-α product. Scaled-up reaction mixtures containing membranes were centrifuged at 125,000 × g for 3 hr to pellet the membrane vesicles. The pellets (containing membranes and ribosomes) were resuspended in buffer A or in the amount of S-100 that was equal to that present in original reaction mixture. These reconstituted reaction mixtures were then incubated in the presence or absence of Triton X-100 for 90 min at 31°C. The reaction mixtures were then supplemented with Triton and deoxycholate and centrifuged to remove ribosomes, and immunoprecipitation of the resulting supernatant fraction was carried out as described (6). Where indicated, yeast mannan was added to give a final concentration of 5 mg/ml.

reaction mixtures, the addition of Triton was necessary for the expression of the presumed mannosidase activity. This suggested that the glycosylated product was protected by microsomal membranes from mannosidase action. Consistent with previous data, mannan blocked the cell-sap-induced conversion in the presence of Triton (Fig. 5). It is curious that in this assay 0.5% Triton was necessary for processing band D, whereas the addition of 0.04% Triton to the translation mixtures was sufficient for the conversion. Perhaps during reisolation the membrane aggregated in some form that was more resistant to low levels of Triton.

DISCUSSION

The data presented here show that a mannose-rich oligosaccharide, which is initially attached to the growing polypeptide chain, undergoes post-translational processing. When Triton X-100 (final concentration 0.04%) was added to the ascites lysate after 60 min of incubation, there was a time-dependent flow of radioactivity from band D to two discrete protein forms, band M and band A. The formation of these proteins was not the result of proteolysis of protein D but was, apparently, due to the action of one or more mannosidases present in the lysates.

Recent studies on the glycosylation of viral proteins *in vivo* have shown that the asparagine-linked nascent oligosaccharide contains di-*N*-acetylchitobiose, 9 or 10 mannose residues, and 1–3 glucose residues (7–9, 21–24). Several workers showed that removal of glucose was an early step in the processing of the nascent sugar unit on the G protein in vesicular stomatitis virus. We have treated band D with yeast α- and β-glucosidase and with a specific liver glucosidase (a gift from M. Michael and S. Kornfeld) and have observed no change in its migration on $NaDodSO_4$ gels. We cannot determine from this experiment if some glucose was present in band D, because it is not clear if the loss of glucose would cause a significant change in the migration of the protein on $NaDodSO_4$ gels.

Inhibition of processing of band D by yeast mannan and purified mannose oligosaccharides support the idea that, *in vitro*, the mannose-rich sugar core in band D was exposed to a mannosidase activity. Because glucose is apparently the terminal sugar of the oligosaccharide core, prior glucose removal may be necessary for mannosidase activity. Therefore, these inhibitors might also interfere with processing of band D by blocking a glucosidase. However, this is unlikely, because there is glucosidase activity in microsomal membranes (24, 25) and therefore glucose was probably removed from band D after the initial 60-min incubation.

Mannosidase activities are present in cytosol, lysosomes, and Golgi membranes (26, 27), and it is conceivable that one or more of these enzymes are involved in the processing of the nascent sugar core. Support for more than one mannosidase was the observation that two discrete proteins were formed in the presence of Triton, suggesting that at least two steps are involved in the processing observed here (Fig. 4). This is consistent with data of Tabas *et al.* (7), who showed that at least two mannosidase activities are required for the trimming of the G_1 protein of vesicular stomatitis virus.

From the data presented it is clear that mannosidase activity was present not in microsomal membranes but rather in the S-100 fraction, which presumably contains enzymes from other subcellular components. For example, because the ascites lysate is prepared in a hypotonic medium, we cannot exclude the possibility that, during preparation, Golgi membranes were disrupted and remained in the cell sap. Furthermore, detergent markedly enhances the activity. Perhaps Triton was required not only for releasing the glycoprotein from within the vesicles of the endoplasmic reticulum but also for solubilizing intracellular mannosidase. Thus, a possible scheme for the *in vitro* processing of the nascent sugar core involves the following events: After the mannose-rich oligosaccharide is attached to the acceptor asparagines, it remains in the same form within the vesicles of the endoplasmic reticulum (presumably glucose residues are split off in the vesicles). Further processing of the released mannose-rich glycoprotein requires exposure to another cellular compartment, where several monosaccharides are removed, leaving the mannose residues that are present in the mature sugar structure. In the present case, this processing was affected by using Triton, which rendered the mannose-rich sugar unit accessible to the action of mannosidase. Thus, the crucial element in this scheme is that trimming is a compartmentalized process in which the removal of mannoses requires a transfer of the glycoprotein from the endoplasmic reticulum to another component of the secretory cascade.

A critical question that arises concerns the structure of the observed intermediates. Kornfeld *et al.* (28) proposed that the removal of sugars from the nascent core of the G protein proceeded in a stepwise manner, resulting in proteins containing nine, five, and three mannose residues. On the basis of the migration of band M and its sensitivity to α-mannosidase from jack bean (data not shown) and to endoglycosidase H, the protein apparently contains at least four mannose residues. The proteins migrating in the region of band A probably represent more extensively processed intermediates, presumably resulting from mannosidase activity. We cannot, as yet, exclude the possibility that some of the forms observed after Triton addition are the result of nonspecific action of one or more cytosolic mannosidases. Thus, the newly synthesized oligosaccharide chain may not normally be exposed to such activity during the transport of the glycoprotein through the secretory pathway.

In any case, this *in vitro* system should permit us to define the physiological significance of the observed intermediates as well as the role of subcellular compartments in the processing of glycoproteins.

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