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ROLE OF CARBOHYDRATE IN GLYCOPROTEIN SECRETION BY HUMAN HEPATOMA CELLS

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SUMMARY: We have previously shown that export of nine proteins by human hepatoma cells falls into three discrete kinetic classes with intracellular retention half-times of approximately 35 min, 77 min and 115 min.[1] To determine if carbohydrate on secretory glycoproteins determines the secretory class we have measured the kinetics of export of the nine proteins after tunicamycin-treatment of cultures. We found no apparent correlation between the kinetic class of a secretory protein and sensitivity of secretion to tunicamycin-treatment. For example, three glycoproteins are exported with rapid kinetics and secretion of only one, α_1-protease inhibitor, is inhibited by tunicamycin treatment. In addition, three glycoproteins are secreted with intermediate kinetics and tunicamycin-treatment inhibits the secretion of two of these proteins, α_2-macroglobulin and ceruloplasmin but not the third, plasminogen.

INTRODUCTION: It is presently unknow what factors regulate the rate of intracellular transport of secretory proteins. We have previously shown that export of nine proteins by a human hepatoma cell line, Hep G2, falls into three discrete kinetic classes: (i) a rapidly-secreted class with an intracellular retention half-time of 30-40 min (albumin, fibronectin, α-fetoprotein and α_1-protease inhibitor), (ii) an intermediate-secreted class with a half-time of 75-80 min (ceruloplasmin, α_2-macroglobulin and plasminogen), (iii) and a slowly-secreted class with an intracellular retention half-time of 110-120 min (fibrinogen and transferrin) (1). Our finding that there are three distinct kinetic classes of secretory proteins suggests that proteins of the same secretory classes share structural determinants which influence their rate of

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export. These structural determinants might include the oligosaccharide structure on glycoproteins or some characteristic feature of their amino acid sequence or conformation.

Carbohydrate is known to have a functional role in intracellular transport of some glycoproteins (2). In many cell types, the major pathway for transport of newly synthesized acid hydrolases to lysosomes requires phospho-mannosyl residues (3) and mutant lymphoma cells deficient in surface expression of specific glycoproteins are defective in oligosaccharide maturation (4). Recently, inhibitors of specific steps in the oligosaccharide processing pathway have been shown to alter intracellular transport of membrane, secretory and lysosomal glycoproteins (5-10).

Treatment of cells with TM, an inhibitor of N-glycosylation of proteins, has been reported to inhibit the secretion of many proteins, but not others (for recent review, see 11). However, interpretation of experiments using TM-treatment of cells can be difficult since removal of carbohydrate can change the solubility properties of proteins, induce aggregation, change the conformation and enhance proteolysis (12-14). In this study we report that TM-treatment of Hep G2 inhibits the rate of export of only three of nine proteins studied and there is no apparent correlation between the kinetic group of the protein and sensitivity of secretion to TM-treatment.

MATERIALS AND METHODS

Materials. Hep G2 cells were a generous gift from Drs. Barbara B. Knowles and David P. Aden, Wistar Institute, Philadelphia, PA. TM was a gift of Dr. Gakuzo Tamura via the Drug Evaluation Branch of the National Cancer Institute of the National Institutes of Health, Bethesda, MD. L-[^{35}S]-methionine (1000 Ci/mmol, carrier free) was obtained from New England Nuclear, Boston, MA., monospecific rabbit antisera against human serum proteins from Accurate Scientific Corp., Westbury, N.Y. Aprotinin from Sigma Chemical Co., St. Louis, MO. and fixed Staphylococcus aureus cells (Pansorbin) from Calbiochem-Behring, San Diego, CA.

Growth and Labeling of Cells. Cultures of Hep G2 cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Confluent monolayer cultures, in 25 cm^2 flasks (Falcon), were labeled with [^{35}S]-methionine by washing three times with phosphate-buffered saline (PBS), then incubating for 10 min in 2.0 ml of serum- and methionine-free medium containing 215 Ci [^{35}S]-methionine at 37°C. Upon completion of labeling, the medium was aspirated, the cells were washed three times with PBS, and serum-free MEM containing 10 mM unlabeled methionine was added. After the appropriate chase times, the medium was collected and put on ice. The cell monolayers were

washed twice with PBS and then 200 µl of additional lysis buffer (1% sodium deoxycholate, 1% Nonidet P-40 and 1% Aprotinin) was added. After 5 min at room temperature, the lysates were collected and the culture flasks washed with 200 µl of additional lysis buffer. Pooled lysate samples were centrifuged at 15,000 x g for 10 min at 5°C to pellet nuclei and cell debris.

Immunoisolations and SDS-PAGE fluorography. Immunoisolations were performed according to the modified procedure of Kessler (15). Media samples were adjusted to 0.1% SDS, 1% Triton X-100, 1% Aprotinin, 25 mM Tris-Cl (pH 7.2) with 10 x stock solution, and lysate samples were adjusted to contain 0.1% SDS, 0.5% sodium deoxycholate and 0.5% Nonidet P-40. Medium (0.75 ml) or cell lysate (0.5 ml) was incubated with 10 µl of the appropriate rabbit antiserum for 1 hr at room temperature, then 20 µl of Pansorbin, which was prewashed in RIPA (0.1% SDS, 1% Triton X-100 in PBS) and ovalbumin (1 mg/ml final concentrations) were added to lysate and media samples and incubated for 30 min at room temperature with continuous rotation. Samples were centrifuged at 1600 x g for 10 min, the pellets were washed three times by centrifugation and resuspension in RIPA-ovalbumin (1 mg/ml) buffer, and the pellets were prepared for SDS-PAGE. In control studies, we obtained essentially quantitative immunoisolation of labeled proteins with this method, and the isolated proteins co-migrated with authentic protein standards on SDS-polyacrylamide gels. SDS-PAGE (7.5% polyacrylamide) was performed according to the Laemmli procedure (16). The gels were fixed, dried and prepared for fluorography as described previously (9, 16, 17).

RESULTS

To evaluate the consequences of oligosaccharide depletion on the secretion of glycoproteins using TM-treatment of cells, it is important to avoid prolonged exposure of cells to the drug to prevent undesirable secondary effects. Therefore, in preliminary experiments, we determined the minimum exposure period of cells to TM required to achieve inhibition of protein glycosylation and we found that preincubation of Hep G2 cells with 10 µg/ml TM for 3 hr prior to pulse-labeling (10 min) with [^{35}S]-methionine was sufficient to inhibit glycosylation by 80-90% without significant inhibition of protein synthesis (fig. 1). As expected, TM-treatment reduced the apparent molecular weights of transferrin, α_1-protease inhibitor, α-fetoprotein, ceruloplasmin, α_2-macroglobulin and the beta and gamma subunits of fibrinogen. The major form of plasminogen contains an O-linked oligosaccharide, hence is insensitive to TM-treatment; however, a subpopulation also contains one biantennary oligosaccharide (19), which is consistent with our finding that TM-treatment slightly reduces the width of the plasminogen band in SDS-polyacrylamide gels (data not shown).

To determine the effect of TM on the kinetics of secretion of the nine proteins, Hep G2 cultures were treated for 3 hr with TM then incubated for

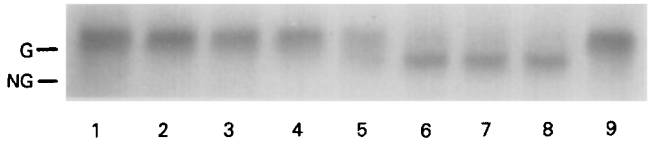

Figure 1. Effect of TM-treatment on the electrophoretic mobility of transferrin. Cultures were incubated with TM (10 µg/ml) for the indicated periods before a 10 min incubation with [^{35}S]-methionine. Transferrin was immuno-isolated and quantitated as described in methods. The lanes shown (from 1 through 9) represent TM-treatment of 0', 30', 60', 120', 150', 180', 210', 240', and an untreated control respectively. G represents glycosylated and NG nonglycosylated.

10 min at 37 C in medium containing [^{35}S]-methionine and TM, washed with PBS and chased for various periods in medium containing an excess of unlabeled methionine without TM. Specific proteins were quantitatively isolated from both the medium and cell lysate by immuno-isolation with monospecific antibodies. We found that TM-treatment markedly inhibits the secretion of α_2-macroglobulin, α_1-protease inhibitor and ceruloplasmin (fig. 2), but had

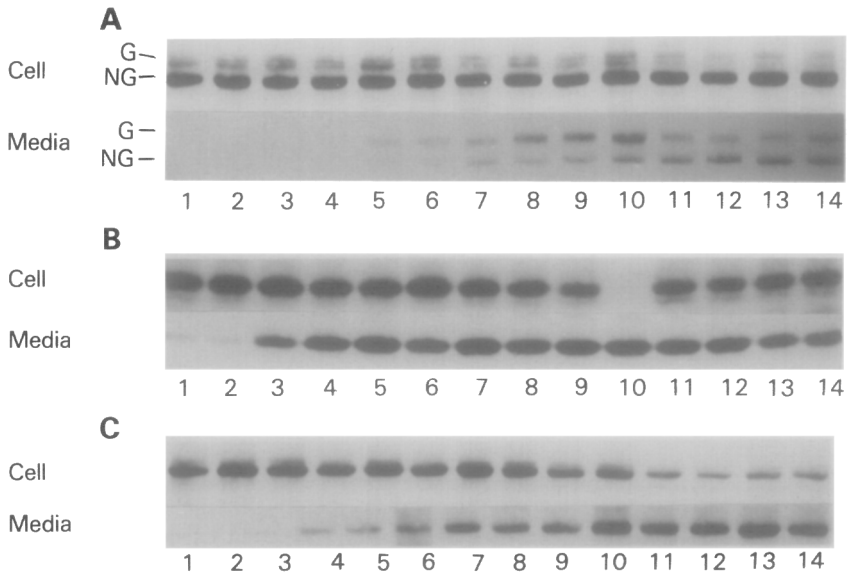

Figure 2. Effect of TM-treatment on the secretion of α_2-macroglobulin, α_1-protease inhibitor and ceruloplasmin. The cells were pretreated with TM (10 µg/ml) for 3 hr, then pulsed with [^{35}S]-methionine for 10 min followed by a chase in growth medium without TM. α_2-macroglobulin (A), α_1-protease inhibitor (B) and ceruloplasmin (C) were immuno-isolated from the cell lysates and media at (1) 0', (2) 15', (3) 30', (4) 45', (5) 60', (6) 75', (7) 90', (8) 105', (9) 135', (10) 165', (11) 225', (12) 285', (13) 345' and (14) 405' after the pulse with [^{35}S]-methionine as described in methods. G represents glycosylated and NG-nonglycosylated α_2-macroglobulin.

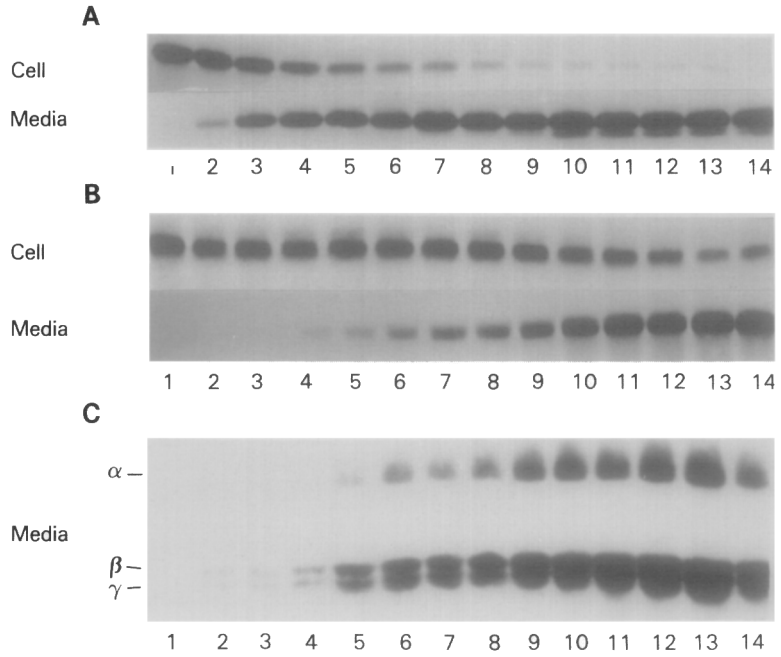

Figure 3. Effect of TM on the secretion of albumin, transferrin and fibrinogen. The experiment was performed as described in legend to Figure 2 and (A) represents albumin, (B) transferrin and (C) fibrinogen.

no effect on the intracellular transport of albumin nor on the glycoproteins transferrin, fibrinogen, α-fetoprotein, fibronectin and plasminogen (fig. 3 and other data not shown).

The most dramatic effect of TM-treatment was on the secretion of α_2-macroglobulin with more than 90% of the protein still present in the cell fraction in an undegraded form almost 7 hr after synthesis, while in control cells, 50% of newly synthesized α_2-macroglobulin is secreted by 75-80 min. A small fraction of α_2-macroglobulin escapes inhibition of glycosylation by TM and migrates more slowly on the SDS-polyacrylamide gel than the major nonglycosylated form, and the glycosylated form is secreted quantitatively during the chase period. Only 40-45% of nonglycosylated α_1-protease inhibitor is secreted during a chase period of almost 7 hr, whereas 50% of the glycosylated form is secreted in 30-40 min in control cells. Half of newly synthesized ceruloplasmin is secreted in 165 min by TM-treated cells compared to 75-80 min in untreated cells. Similar kinetics of secretion for the respective proteins were obtained using either data on the loss of proteins

from the cell or accumulation of proteins in the medium, and the rates were highly reproducible in four separate experiments. Proteolysis was minimal in these experiments since there was no significant loss of total (cell lysate + media) (^{35}S)-methionine cpm incorporation into these proteins during the chase periods.

DISCUSSION

TM-treatment of Hep G2 cultures inhibits the rate of secretion of three glycoproteins, α_1-protease inhibitor, ceruloplasmin and α_2-macroglobulin, but does not alter the kinetics of secretion of albumin, α-fetoprotein, fibronectin, plasminogen, fibrinogen and transferrin. There is no apparent correlation between the kinetic class of a secretory protein and sensitivity of secretion to TM-treatment. For example, of the three glycoproteins that are exported with rapid kinetics secretion of only one of these, α_1-protease inhibitor, is inhibited by TM-treatment. Similarly, of the three glycoproteins secreted with intermediate kinetics TM-treatment inhibits the secretion of only two of these proteins, α_2-macroglobulin and ceruloplasmin but not the third, plasminogen. These results agree with previous reports that TM-treatment of hepatocytes and hepatoma cells inhibits secretion of α_1-protease inhibitor but not albumin or transferrin (7, 20, 21).

It is presently unclear why TM-treatment inhibits secretion of specific glycoproteins. Removal of carbohydrate by TM-treatment has been shown to alter the conformation of some glycoproteins and induce the formation of insoluble aggregates (12, 13) which could inhibit intracellular transport of proteins. While we cannot exclude this possibility, we now know that the carbohydrate-depleted proteins, whose secretion is disrupted by TM-treatment of Hep G2 cultures, are released in a soluble form after cell disruption by freeze-thaw (T.-K. Yeo unpublished data).

The present results suggest an apparent correlation between the presence of specific carbohydrate structure on secretory proteins and sensitivity of their secretion to TM-treatment. Both ceruloplasmin and α_1-protease inhibitor from human sources are known to have at least one triantennary

complex oligosaccharide (22, 23) and α_2-macroglobulin also has triantennary, oligosaccharides based on indirect evidence (24, 25). However, the five glycoproteins that are insensitive to TM inhibition of secretion lack triantennary forms and most have biantennary, complex oligosaccharides (19, 26-29). Recently TM treatment of Hep G2 has been shown to inhibit secretion of thyroxine-binding globulin (30) and this glycoprotein also possesses triantennary complex oligosaccharides (31) further supporting the correlation. These results suggest that triantennary complex forms or oligosaccharide precursors of these forms may be required for export of specific glycoproteins in Hep G2 cells. However, the carbohydrate structures were determined mostly using protein isolated from human serum and it is possible that the glycan moieties are not similar in Hep G2 cells due to alterations associated with transformation (32)---a consideration currently under investigation.

Although our results show that carbohydrate is not the main determinant which specifies the kinetics of secretion of many glycoproteins, there is evidence that the structure of the oligosaccharide does have some regulatory function in the secretory pathway(s). For example, modification of oligosaccharide structure by treatment of cells with swainsonine or deoxynojirimycin inhibitors of specific steps in the oligosaccharide processing pathway (33), alters the rate of intracellular transport of glycoproteins. Swainsonine treatment of Hep G2 accelerates post ER transport of secretory glycoproteins (9) and deoxynojirimycin treatment delays their transport from the RER to the Golgi (7, 10).

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