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THE POLY-*N*-ACETYLLACTOSAMINE CHAINS IN GLYCOPROTEINS SYNTHESIZED BY CHINES HAMSTER OVARY CELLS AT 20° AND 37°HAVE SIMILAR STRUCTURES

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We have found recently that many of the poly-*N*-acetyllactosamine sequences in glycoproteins synthesized by Chinese hamster ovary (CHO) cells contain terminal sialic acid residues linked ∝ 2, 3 to penultimate galactose residues. However, we also found that in the wheat germ agglutinin-resistant CHO cell line clone 1021, which is unable to sialylated either its glycoproteins or glycolipids, the poly-*N*-acetyllactosamine chains are the same length and overall size as those from CHO cells. These results suggests strongly that sialylation of poly-*N*-acetyllactose-amine chains in glycoproteins does not serve to limit the length of newly-synthesized chains. To investigate other factors that might influence the biosynthesis of these chains, we have analyzed the effect of low temperature on the structures of newly synthesized poly-*N*-acetyllactosamine chains. Reports by other investigators have demonstrated that at 20°, newly synthesized glycoproteins in animal cells are sequestered in the Golgi apparatus and terminally glycosylated; however, at this temperature transport of glycoproteins to the cell surface is blocked. We have found that the radiolabeled glycopeptides derived from CHO and glycoproteins synthesized in cells at 20° in the presence of [6-H^3]·galactose are similar in length and to those made at 37°; however, at 20° most of these glycoproteins are not expressed on the surface of the cells. These results demonstrate that low temperature block of glycoprotein translocation in cells does not result in substantial alterations in the structures of newly synthesized poly-*N*- acetyllactosamine chains.

Key words: Poly-*N*-acetyllactosamine, Effect of temperature, Glycoproteins

INTRODUCTION

The factors regulating the biosynthesis of animal cell glycoproteins are not completely understood. Although it is clear that glycosyltransferases and glycosidases in the endoplasmic reticulum and Golgi apparatus are required for glycosylation of proteins Kornfeld [1], other factors, such as protein conformation and translocation rates through intracellular organelles, may also be important in determining the structures of the oligosaccharide moieties (Regoeczi, *et.al.* [2], Snider and Rogers [3], Griffiths and Simons [4].

It has recently been shown that low temperature can reversibly alter the translocation of glycoproteins through intracellular organelles. At 15° glycoproteins exit the endoplasmic reticulum but are sequestered in a pre-Golgi, apparatus region (Saraste and Hedman [5]), Saraste and Kuismanen [6], however, at 20°, glycoproteins move through the Golgi apparatus and are terminally glycosylated, but the glycoproteins do not exist to the cell surface (Matlin and Simons [7]), Griffiths, *et. al.* [8]. At 20° glycoproteins appear to accumulate in what is termed the trans-golgi network (Griffiths and Simons [4]), which appears to represent the site of sorting for new synthesized glycoproteins. Interestingly, there is evidence that the newly synthesized G-protein of vesicular stomatitis virus-infected MDCK cells, is more sialylated at 20° that at 37° (Fuller, Bravo and Simons [9]).

We recently demonstrated that surface glycoproteins in the Chinese hamster ovary (CHO) cell line contain poly-N-acetyllactosamine sequences and that about 1/2 of these sequences contain terminal β-linked galactose residues and the remainder contain sialic acid in ∝-2,3 linkage to penultimate galactose. We investigated the role of sialylation in regulating the length of the poly-*N*-acetyllactosamine chains, by examining their structures in a wheat germ agglutinin-resistant Clone of CHO, designated Clone 1021. These cells are unable to sialylate either their glycoproteins or glycolipids (Briles, *et. al.*, [10]), because of a defect in their ability to import CMP-NeuAc into the Golgi apparatus Deutscher, *et. al.* [11]. Our results demonstrated that the poly-*N*-acetyllactosamine chains synthesized by Clone 1021 cells are not sialylated, but are otherwise similar in length to those synthesized in CHO cells. These results demonstrated that sialylation has little, if any, influence on the length of newly synthesized poly-*N*-acetyllactosamine chains.

We have investigated the effect of low temperature on

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the biosynthesis of glycoproteins in CHO and Clone 1021 cells. Our results demonstrate that at 20° newly synthesized glycoproteins are sequestered intracellularly and that the poly-*N*-acetyllactosamine chains are similar in length to those synthesized at 37°.

EXPERIMENTAL

Materials. D-[6-H^3] galactose was purchased by INC and Sephadex G-25 and G-50 was obtained from Sigma. Trypsin was purchased from Flow Laboratories, Inc. and Pronase was obtained from CalBiochem. All materials for polyacrylamide gel electrophoresis and the molecular weight standards were purchased from BioRad, Inc. *Escherichia freundii* endo B-galactosidase was kindly supplied by Dr. Minoru Fukuda (Cancer Research Center, La Jella Cancer Research Foundation, La Jolla, CA). The standard oligosaccharides, GlcNAc β1, 3Gal; Gal β1, 4G1cNAc β1, 3Gal; and Sia 2,3Gal β1, 4G1cNAc β1, 3Gal were derived as described previously (Merkle and Cummings [12].

Preparation of [H^3]-galactose-labeled glycopeptides from cultured cells. Chinese hamster ovary (CHO) cells and Clone 1021 cells were cultured routinely in 100 mm tissue culture plastic dishes from Falcon in alpha-minimal essential medium containing 10% fetal calf serum and 100 1U/m1 penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Cells were metabolically radiolabeled with H^3-galactose either in culture on dishes or after removal from dishes in suspension. When cells were radiolabeled in dishes, the dishes were covered and floated on a water bath at the set temperature. In most cases 50 to 200 µCi of H^3-galactose was dried under nitrogen and resuspended in complete media, containing 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid), pH 7.4. This was added directly to cells incubating at either 20° or 37°.

Cells were trypsinized to remove them from dishes using 0.025% trypsin in Hanks salts buffer at room temperature for 1 minute. The cells were triturated from the dish and washed twice in complete medium containing 10 mM HEPES.

At the end of metabolic radiolabeleling the cells were either; washed twice on the dish in PBS (phosphate-buffered saline-6.7 mM KH_2PO_4 and 150 mM NaC1, pH 7.4) and scraped from the dish using a rubber policeman into 0.5 ml of PBS; or centrifuged for 5min. at 500xg. In the latter case the labeling medium was removed and the cells were washed twice 5 ml PBS in the tube by centrifugation.

Column chromatography The gel filtration chromatography of glycopeptides on Sephadex G-25 and Sephadex G-50 was conducted on column in 0.1 M pyridine-acetate, pH 5.6.

Treatment with endo-B-galactosidase. Glycopeptides were treated with *E. freundii* endo-B-galactosidase in 0.1 M sodium acetate, pH 5.6, containing 2.4 milliunits of enzyme and 20 micrograms of bovine serum albumin in a final volume of 0.02 ml at 37° for 24 hr. in a tolune atmosphere.

Paper chromatography. Descending paper chromatography of oligosaccharides was performed on Whatman No.1 paper in the solvent system athyl acetate: pyridine: glacial acetic acid: water (5:5:1:3) for 22 hr. (Merkle and Cummings [12]).

Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE). After metabolic radiolabeling, cells were washed and boiled in SDS-sample buffer and radiolabeled glycoproteins were separated in 6 to 18% gradient acrylamide slab gels (14 x 8.5 x 0.15 cm) containing 0.1% SDS Laemmli, [13]. Gels were calibrated with molecular weight standards from Bio-rad. The dried gels were then fluorographed to locate radiolabeled bands. Soderquist and Carpenter [14].

The radiolabeled proteins in the running gel were removed by cutting out the entire lane and the gel was cut into small 2 mm pieces. The pieces were treated with 10 mg/ml pronase in 100mM tris-HC1, 1 mM $CaCl_2$, pH 3.0 at 60° for 12 hr. The sample was then boiled and the released, radiolabeled glycopeptides were recovered in the supernatant after a brief centrifugation at 100 xg. The gel pieces were then washed with 5 ml of water and the samples were combined.

RESULTS

Analysis of poly-N-acetyllactosamine chains in glycoproteins synthesized at either 20°Cor 37°. Preliminary experiments indicated that at 37°, CHO cells incorporated [6-H^3] galactose into glycoproteins and that greater than 95% of incorporated label was recoverable as authentic galactose. However, at 20°, we found that greater 80% of the precursor incorporated into material voided on a Sephadex G-25 column was recoverable as radiolabeled glucose and the remainder was galactose. This H^3-glucose-labeled material synthesized at 20° was not precipitated by trichloroacetic acid or acetone and was also contained in the void volume of a Sephadex G-100 column. These results indicated that at 20° most of the H^3-galactose precursor was diverted into glycogen (manuscript in preparation).

To cleanly separate the glycoproteins from glycogen at the two different temperatures, radiolabeled glycoproteins from the cells were isolated by SDS/PAGE. Confluent cultures of Clone 1021 cells were incubated in dishes (35 mm diameter) at either 37° or 20° for 3 hr. in media containing (6-H) galactose. For the 37° labeling 50 µCi of radiolabeled galactose and for the 20° labeling 200 µCi was used in a total volume of 0.5 ml. After the incubation, the labeling media was removed and the cells were scraped from the dish, washed in PBS and then boiled for 5 min. in SDS-sample buffer. A portion of the solubilized material was precipitated with 10% trichloroacetic acid to assess the incorporation of radioactivity into glycoproteins. Approximately 1.5

million cpm of material was precipitated from the 20° labeled cells and 1.2 million cpm was precipitated from the 37° labeled cells. Since the cells at 20° were incubated with 4 times as much radiolabel, it can be estimated that incorporation into TCA-precipitable material at 20° was only about 1/4 that incorporated at 37°.

The solubilized material was analyzed by SDS/PAGE and H^3-galactose labeled glycoproteins were detected by autoradiography (Fig. 1). Numerous bands of glycoproteins were radiolabeled at both temperatures; however, there were no obvious differences in the electrophoretic migrations of glycoproteins synthesized at either 20° or 37°.

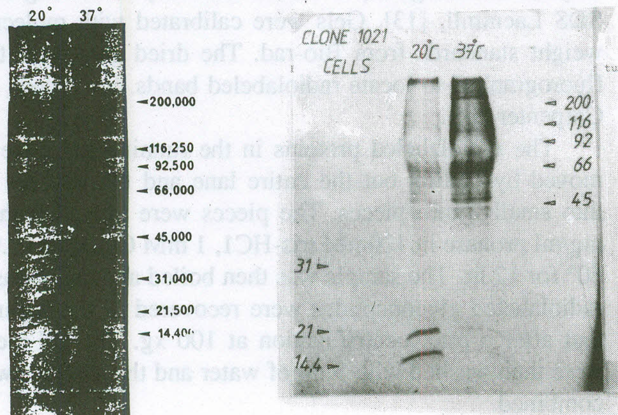

Fig. 1. SDS/PAGE of H^3-galactose-labelled glycoproteins from Clone 1021 cells synthesized at either 20° or 37°. Cells were metabolically radiolabeled as described in experimental procedures and labelled glycoproteins were separated in a 6 to 18% acrylamide slab gel in SDS and fluorographed. The lanes are indicated and molecular weight markers are shown.

To analyze the structures of the poly-*N*-acetyllactosamine chains on glycoproteins synthesized at the two temperatures, the entire lane of radiolabeled glycoproteins were excised from the gel. The lanes were cut into fine pieces and treated exhaustively with 10mg/ml Pronase, to generate radiolabeled glycopeptides. The released glycopeptides were applied directly to a column of Sephadex G-50 and the elution profiles of radiolabeled glycopeptides synthesized at the two different temperatures were indistinguishable (Fig. 2). Portions of the glycopeptides synthesized at 20° and 37° were hydrolyzed in strong acid and greater than 95% of the radioactivity was recovered in authentic H^3-galactose.

To analyze for the presence and structure of the poly-*N*-acetyllactosamine sequences, glycopeptides were treated with endo-β-galactosidase from *E. freundii*. This enzyme cleaves poly-*N*-acetyllactosamine chains at internal galactose residues to release small-sized fragments. For example, the oligosaccharide Sia ∝ 2, 3Gal β1, 4G1cNAc β1, 3Gal B1, 4 GlcNAc β1, 3Gal β1, 4G1cNAc-R can be cleaved by endo-β-galactosidase to release the tetrasaccharide Sia 2, 3Gal β1, 4G1cNAc β1, 3Gal and the disaccharide G1cNAc β1, 3Gal and the residual glycopeptide G1cNAc-R.

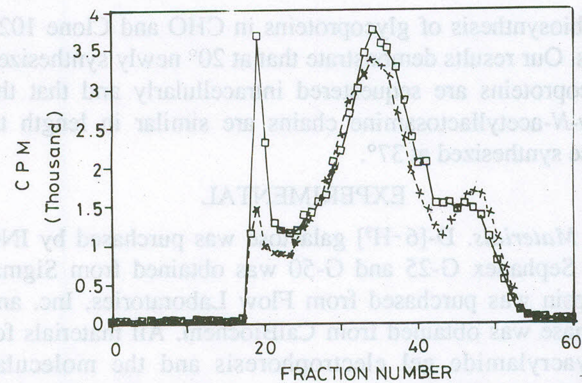

Fig. 2. Sephadex G-50 column chromatography of H^3-galactose-labelled glycopeptides prepared from labeled glycoproteins synthesized by Clone 1021 cells at either 20° or 37°. Radiolabelled glycopeptides were prepared by pronase digestion of the radiolabeled glycoprotein bands from Fig.1, as discussed in the text. Material from 37°(x-x); material from 20°(□ -□)

When the ^3H-galactose-labeled glycopeptides synthesized by CHO cells at either 20° or 37° were treated by endo-β-galactosidase, greater than 50% of the radioactivity was released as two major oligosaccharides, designated A and B (Fig. 3). For the 37° labeled material Peak A contained 34% and Peak B contained 16% of the released radioactivity; for the 20° labeled material Peak A contained 35% and Peak B contained 15% of the released radioactivity. Peak A comigrated with standard Gal β1, 4G1cNAc β1, 3Gal and Peak B comigrated with G1cNAc β1, 3Gal. These are the expected products from H^3-galactose-labeled glycopeptides synthesized by CHO and Clone 1021 at 37°, as shown in our previous study. The observation that identical profiles of released material was recovered from glycopeptides synthesized at 20° demonstrates that the poly-*N*-acetyllactosamine sequences on newly synthesized glycoproteins at this temperature are similar to those synthesized at 37°.

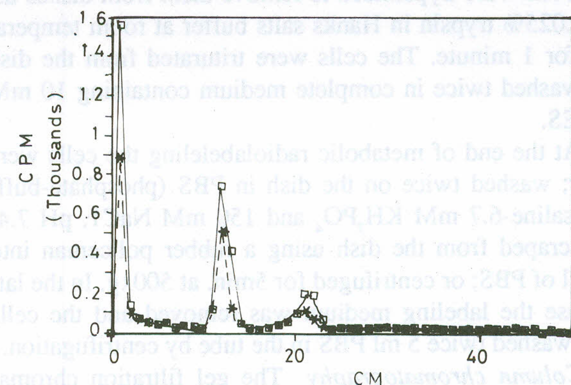

Fig. 3. Descending paper chromatography of H^3-galactose-labelled glycopeptides synthesized by Clone 1021 cells at either 20° or 37° after treatment with *E. freundii* endo-β-galactosidase. Glycopeptides were treated with the enzyme for 24 hr. and analyzed by descending paper chromatography as described in experimental procedures. The origin cpm represent residual, labelled glycopeptide. The slow migrating peak comigrates with the standard Galβ1, 4 G1cNAcβ1, 3 G1cNAcβ 1,3 Gal. Material from 37° (□ -□); meterial from 20° (*-*).

Effect of temperature shift from 20° on the translocation of H^3-galactose-labeled glycoproteins. To determine whether the H^3-galactose-labeled glycoproteins synthesized at 20° were intracellular or on the cell surface, we analyzed the ability of trypsin-treatment on intact cells to release radiolabeled glycopeptides. CHO cells were incubated for 1 hr. in suspension with H^3-galactose in complete medium at either 20° or 37°. The cells were then washed and chased at the original incubation temperature of either 20° or 37°. At times during the chase period, portions of the cells were removed, and treated with 0.02% trypsin in Hanks buffered salts for 10 min. at 20°. The released glycopeptides were recovered in the supernatant after centrifugation of the cells. The released material was desalted on a column of Sephadex G-25 to recover authentic radio-labeled and released glycopeptides. As shown in Fig. 4, significant amounts of radiolabeled glycopeptides were released by trypsin treatment of cells metabolically-labeled at 37°; whereas; only small amounts of material was released from cells labeled at 20°. However, when cells in the 20° labeling were shifted to 37°, increasing amounts of radiolabeled glycopeptides were released. These results demonstrate that at 20° most of the radiolabeled glycoproteins are intracellular and inaccessible to trypsin, whereas upon shifting to 37° significant amounts of these labeled glycoproteins move to the cell surface and become accessible to trypsin.

Interestingly, we found that, upon shifting to 37° after longer times at 20° during the chase period, the efficiency of translocation of radiolabelled glycoproteins to the cell surface was diminished. This might suggest that the viability of cells at 20° is impaired. To examine for this possibility, we incubate cells with H^3-galactose in medium at 20° for up to 6 hr. and found that the incorporation of radiolabel into glycoproteins was entirely linear during this period. These results indicate that the cells are viable and metabolically active at 20°. The observed impairment in the cells ability to translocate glycoproteins to the surface during the 20° to 37° shift is probably not due to cell death or a gradual slowing of cellular metabolism.

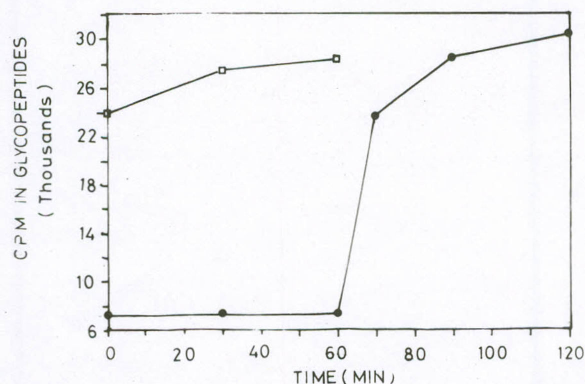

Fig. 4. Effect of temperature on the expression of newly synthesized H^3-galactose-labelled glycoproteins in CHO cells. Cells were incubated in suspension at either 20° or 37° with H^3-galactose in complete medium for 2 hr. The cells were washed and then incubation was continued at the same temperature. After one hour the cells that were chased at 20° were shifted to 37° and the incubation continued. At times cells in the chase were removed both master tubes and washed twice at 4° in 10 mM HEPES in Hanks salts buffer. The washed cells were incubated with 0.025% trypsin in Hanks for 10 min. at 20° to release surface glycopeptides. The released material was recovered in the supernant after centrifugation. The released glycopeptides were recovered in the excluded fractions upon chromatography on a column of Sephadex G-25. The numbers on the ordinate represent the radioactivity in excluded glycopeptides. 37° chase (□-□); 20° chase and shift to 37° (●-●).

DISCUSSION

In this study we have shown that the poly-*N*-acetyllactosamine chains of CHO glycoproteins newly synthesized at either 37° or at 20° are similar in size and sequence these glycoproteins synthesized at 20° sequestered intracellularly and can be exported to the cell surface quantitatively upon warming the cells to 37°.

These results indicate that glycoproteins accumulated in cells at 20° are glycosylated similarly to those made at 37°. Several studies indicate that at 20° glycoproteins accumulate in the transgolgi network (Griffiths and Simons, [4] and that the accumulated glycoproteins can be exported to the surface upon shifting the temperature to 37°. Interestingly, several experiments in other systems indicate that the trans-golgi network contains sialytransferases which can resialylate experimentally-desialylated surface glycoprotein receptors. If indeed glycoproteins are sequestered in the trans-golgi network at 20°, then our results indicate that no further elongation of the poly-*N*-acetyllactosamine chains occurs in this compartment. It is possible that the conformation of glycoproteins in CHO cells limits their accessibility to glycosyltransferases, or that the rate of translocation through the Golgi apparatus is not substantially affected by low temperature. future studies will be aimed at discerning the basis for this phenomenon.

In our other studies we found that the poly-*N*-acetyllactosamine chain length was independent of whether the chains contained terminal sialic acid, Cummings and Cummings [15]. These results suggest that sialyltransferase is not a cometing glycosylation event with the elongation of the poly-N-acetyllactosamine chain. Thus, it is possible that in CHO cells the $\propto$ 2, 3 sialyltransferase is localized in a compartment of the Golgi apparatus distinct from that in which the poly-*N*-acetyllactosamine chain backbone is synthesized.

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