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Articles

Glycosylation and Processing of High-Mannose Oligosaccharides of Thyroid-Stimulating Hormone Subunits: Comparison to Nonsecretory Cell Glycoproteins†

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ABSTRACT: Thyroid-stimulating hormone (TSH) subunit glycosylation was compared to that of total cell glycoproteins in mouse thyrotropic tumors. Lipid-linked oligosaccharides, total cell glycoproteins, and TSH subunits were labeled with either [^{3}H]mannose, [^{3}H]galactose, or [^{3}H]glucose in pulse and pulse–chase experiments. The various oligosaccharides were isolated respectively by lipid extraction and mild acid hydrolysis, by selective immunoprecipitation, or by acid precipitation followed by trypsin and endoglycosidase H treatment. The nature of the oligosaccharides was assessed by their migration in paper chromatography, their relative incorporation of different precursors, and also their resistance to α-mannosidase. At 60 min, lipid-linked oligosaccharides were found to be composed of $Glc_{3-2}Man_9GlcNAc_2$, $Man_{9-8}GlcNAc_2$, and $Man_5GlcNAc_2$. At 10 or 60 min of labeling, total cell proteins contained $Glc_3Man_9GlcNAc_2$, $Glc_1Man_9GlcNAc_2$, $Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, $Man_8GlcNAc_2$, and $Man_7GlcNAc_2$. The largest oligosaccharide, $Glc_3Man_9GlcNAc_2$, had an unusually long half-life of about 2 h. In contrast, no $Glc_3Man_9GlcNAc_2$ was found either on TSH + α subunits or on free β subunits isolated either by immunoprecipitation or by sodium dodecyl sulfate gel electrophoresis. Instead, primarily $Man_9GlcNAc_2$ was found after a 10-min pulse both on TSH + α subunits and on β subunits. When the pulse was followed by a chase up to 2 h, there was a progressive increase in $Man_8GlcNAc_2$ in higher amounts on TSH + α-subunit carbohydrate chains than on β subunits. In addition, when the chase was performed in the presence of hypothyroid serum, a species comigrating with $Glc_1Man_9GlcNAc_2$ was detected primarily on TSH + α subunits. These data suggest a differential carbohydrate processing rate for secretory TSH subunits compared to certain cell nonsecretory glycoproteins and also demonstrate a differential processing rate between TSH + α subunits compared to free β subunits.

The participation of lipid-linked oligosaccharides as intermediates in the biosynthesis of asparagine-linked oligosaccharides has now been well documented (Hubbard & Ivatt, 1981; Struck & Lennarz, 1980; Chapman et al., 1979). On the lipid carrier are preassembled oligosaccharides as large as $Glc_3Man_9GlcNAc_2$, certain ones of which are then transferred en bloc to nascent polypeptide chains (Kiely et al., 1976; Glabe et al., 1980). On the basis of primarily cell-free studies involving total cell glycoproteins (Turco et al., 1977; Spiro et al., 1979), it has been proposed that $Glc_3Man_9GlcNAc_2$ is the common precursor for all asparagine-linked complex and high-mannose-type oligosaccharides of mature glycoproteins. However, recent in vivo studies have shown that in two species of trypanosomes (Parodi et al., 1981; Parodi & Cazzulo, 1982) as well as in concanavalin A resistant mutant Chinese hamster ovary (CHO)[1] cells (Krag, 1979) deficient in the glucosylation of oligosaccharide lipids, $Man_7GlcNAc_2$ or $Man_9GlcNAc_2$ accumulates on lipids without blockade of protein glycosylation. It is unclear at the present time whether the transfer of glucose-free oligosaccharides actually occurs in such cells or in other cells under normal physiologic conditions. A minor glycosylation pathway involving even a smaller oligosaccharide, $Glc_3Man_5GlcNAc_2$, has also been proposed (Rearick et al., 1981).

In VSV-infected CHO cells (Kornfeld et al., 1978) and in chick embryo fibroblasts (Hubbard & Robbins, 1979), asparagine-linked oligosaccharides were shown to be subse-

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[1] Abbreviations: CHO, Chinese hamster ovary; CG, chorionic gonadotropin; Con A, concanavalin A; endo H, endo-β-*N*-acetylglucosaminidase H; SDS, sodium dodecyl sulfate; TSH, thyroid-stimulating hormone; VSV, vesicular stomatitis virus; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

quently trimmed of all three glucose residues and six of their nine mannose residues prior to conversion to complex-type structures. Whether secretory and nonsecretory glycoproteins are processed through the same pathway is unknown. Moreover, no definite information has been obtained regarding the mechanism by which certain oligosaccharides remain as high-mannose forms.

Thyroid-stimulating hormone (TSH) is composed of two glycosylated, noncovalently linked subunits, α and β. TSH-α contains two asparagine-linked oligosaccharides whereas TSH-β has only one (Pierce & Parsons, 1981). Within a species, the α subunit of TSH is virtually identical with that of the pituitary hormones lutropin and follitropin and the placental chorionic gonadotropin (CG), and the biological specificity of these hormones resides in their β subunits (Pierce & Parsons, 1981). Recent investigations from this laboratory have shown that TSH glycosylation is initiated with high-mannose oligosaccharides (Magner & Weintraub, 1982), the latter being necessary for attainment of proper subunit conformation (Giudice & Weintraub, 1979), for α–β subunit combination, and for protection from intracellular degradation and aggregation (Weintraub et al., 1983). In view of these observations, the present study was directed toward the elucidation of the early glycosylation and carbohydrate processing of TSH subunits as an example of a secretory polymeric glycoprotein. Oligosaccharides from TSH α and β subunits were compared to those from lipid precursors and to those from total cell as well as selected individual nonsecretory proteins. Our results suggest differences in the rates of carbohydrate processing between TSH subunits themselves as well as between TSH and other cell glycoproteins. The regulation of TSH subunit glycosylation of hormonal and metabolic factors has also been studied[2] and will be published elsewhere.

Materials and Methods

Materials. D-[2-^{3}H]Mannose (13.4 Ci/mmol), D-[1-^{3}H]-galactose (8.2 Ci/mmol), and D-[2-^{3}H]glucose (14.1 Ci/mmol) were from Amersham/Searle. Endoglucosaminidase H was kindly provided by Dr. F. Maley (New York State Department of Health, Albany, NY). Trypsin and α-mannosidase (type III) were from Sigma. All other chemicals were of analytical grade from commercial suppliers.

Incubation of Tumor Minces. Mouse pituitary thyrotropic tumors were induced and transplanted as previously described (Blackman et al., 1978). One to four tumors (average weight 3 g) were minced and preincubated for 30 min at 37 °C in 5% CO_2–95% air in sterile tubes containing glucose-free and serum-free Dulbecco's-modified Eagle's medium supplemented with 2 mM glutamine. In continuous-labeling experiments, tumor minces were incubated in serum-free medium containing 0.1 mg/mL glucose and 2 mM glutamine in the presence of 500 μCi/mL [^{3}H]mannose, [^{3}H]glucose, or [^{3}H]galactose for either 2, 5, 10, or 60 min. Incubations were terminated by washing with an excess of cold medium. In pulse–chase experiments, minces were incubated for 10 min in the presence of [^{3}H]mannose or [^{3}H]galactose and centrifuged at 1000g for 2 min followed by removal of the supernatant and further incubation of the minces for 60 or 120 min in the presence of 1 mg/mL glucose plus 1 mM unlabeled sugar. At the end of the incubation, minces were centrifuged at 1000g for 2 min, media were removed, and the pellets were washed once, frozen, and stored at −20 °C.

Oligosaccharide Lipids. Immediately after being labeled, the minces were harvested in chloroform–methanol–water (3:2:1 v/v) and delipidated as previously described (Ronin & Bouchilloux, 1976). Oligosaccharide lipids were selectively extracted by the chloroform–methanol–water mixture (10:10:3 v/v) and the oligosaccharides released from lipids by mild acid hydrolysis (Ronin & Bouchilloux, 1978).

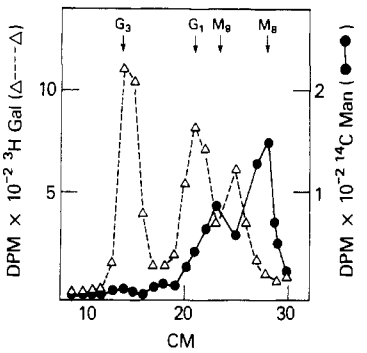

FIGURE 1: Paper chromatography of endo H released oligosaccharides. Separation of [^{3}H]galactose-labeled oligosaccharides in the presence of [^{14}C]mannose-labeled $Man_9GlcNAc$ and $Man_8GlcNAc$ internal standards. The chromatogram was run for 48 h as described under Materials and Methods. CM in this and subsequent figures indicates the number of centimeters from the origin of the paper chromatogram.

Total Cell Glycoproteins. Tumor minces were thawed and homogenized in 0.15 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.01 M EDTA, 1% Triton (w/v), and 1 mM mannose and glucose. After sonication, the lysates were centrifuged at 100000g for 2 h. Aliquots of lysates were precipitated by 10% trichloroacetic acid–1% phosphotungstic acid (w/v), washed twice, extracted twice by acetone–ether (1:1 v/v), and redissolved in 200 μL of 0.06 M ammonium bicarbonate. Trypsin (5% w/v) was added, and the specimens were incubated for 16 h at 37 °C, lyophilized, redissolved in 75 μL of 0.1% SDS, 1% mercaptoethanol, and 0.01 M sodium citrate, pH 5.5, boiled, and finally treated with endo H (5–10 mIU) in 20 mM sodium citrate, pH 5.5, for 16 h at 37 °C. Control experiments have indicated that this method yielded results comparable to those from our previously published method of protein delipidation followed by Pronase digestion (Ronin & Bouchilloux, 1976, 1978).

TSH Subunits. The lysates were first precipitated with specific rabbit anti-bovine LH-α using a staphylococcal protein A method described previously (Weintraub et al., 1980). This procedure has been shown to precipitate both uncombined α and combined α and β in TSH. Then the supernatants were reprecipitated with anti-bovine TSH-β to isolate uncombined TSH β subunits.

Immune complexes were eluted from protein A by boiling in 0.06 M ammonium bicarbonate for 5 min. Trypsin was subsequently added, and the samples were digested for 4 h at 37 °C, lyophilized, and further incubated with SDS, mercaptoethanol, and endo H as described above.

Paper Chromatography. The size of endo H released oligosaccharides was estimated by chromatography on Whatman 1 paper using 1-propanol–nitromethane–water (5:2:4) as the developing solvent (Staneloni et al., 1980) for either 16, 24, or 48 h. Each ^{3}H-labeled sample was run with selected ^{14}C-labeled internal standards as shown in Figure 1. The latter were obtained from total proteins labeled with [^{14}C]mannose as described above and characterized by comparison to well-defined ^{3}H-labeled high-mannose oligosaccharides kindly provided by Drs. Stuart Kornfeld (Washington University, St. Louis, MO), Phillips Robbins (Massachusetts Institute of Technology, Cambridge, MA), Sam Turco (University of Kentucky, Lexington, KY), and Frank Maley (State of New York, Department of Health, Albany, NY). Glucose-con-

[2] See Ronin et al. (1983).

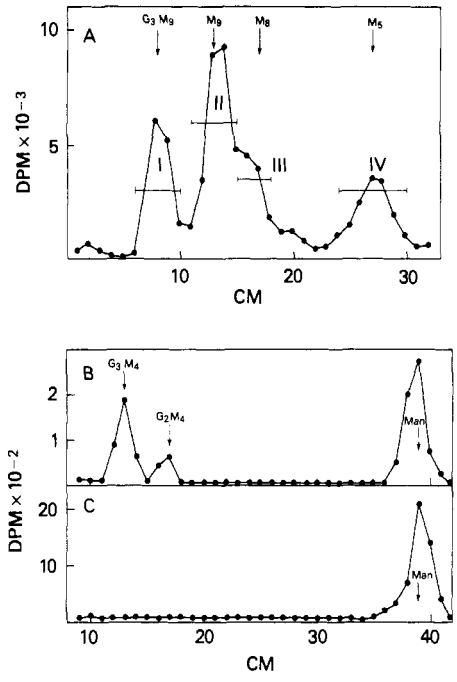

FIGURE 2: [^{3}H]Mannose-labeled lipid-released oligosaccharides from thyrotropic tumor minces incubated for 60 min. (A) The oligosaccharides were subjected to paper chromatography for 24 h. (B and C) The separated oligosaccharides were eluted as noted, digested with α-mannosidase, and rechromatographed for 16 h. The chromatogram for peak I is shown in panel B, while that for peaks II–IV is shown in panel C. The standards are $Glc_3Man_9GlcNAc_2$ (G_3M_9), $Man_9GlcNAc_2$ (M_9), $Man_8GlcNAc_2$ (M_8), $Man_5GlcNAc_2$ (M_5), $Glc_3Man_4GlcNAc_2$ (G_3M_4), and $Glc_2Man_4GlcNAc_2$ (G_2M_4).

taining oligosaccharide standards were prepared from porcine thyroid glands and have been characterized previously (Ronin et al., 1981).

Polyacrylamide Gel Electrophoresis. Delipidated proteins (Ronin & Bouchilloux, 1976) were analyzed by SDS–polyacrylamide gel electrophoresis as previously described (Giudice & Weintraub, 1979). Gel slices (1 mm) were eluted with 0.5 mL of 0.06 M ammonium bicarbonate for 36 h at 37 °C. The eluted proteins were then precipitated with cold acetone (8:1 v/v) in the presence of 1 mg of bovine serum albumin and subsequently treated with trypsin and endo H as described above.

Treatment with α-Mannosidase. Labeled oligosaccharides were incubated for 16 h at 37 °C in 100 μL of 20 mM acetoacetic acid, pH 4.5, in the presence of α-mannosidase (2.6 units) previously dialyzed for 5 h against the incubation buffer. At the end of the incubation, the samples were boiled for 3 min and analyzed by paper chromatography as described above.

Radioactivity Measurements. Paper strips (1 × 3 cm) were eluted for 2 h with 1 mL of water, and liquid scintillation counting was performed by using 10 mL of a detergent-containing solution (Ultrafluor, National Diagnostics) as previously described (Weintraub et al., 1983).

Results

Characterization of Lipid-Linked Oligosaccharides. After incubation of tumor minces with [^{3}H]mannose for 60 min, lipid extraction, and mild acid hydrolysis, several oligosaccharides were released from the lipid carrier and separated by paper chromatography as shown in Figure 2. Four oligosaccharides of different molecular size were found comigrating respectively with $Glc_3Man_9GlcNAc_2$, $Man_{9-8}GlcNAc_2$, and $Man_5GlcNAc_2$ standards (Figure 2A). Each peak was eluted from the paper

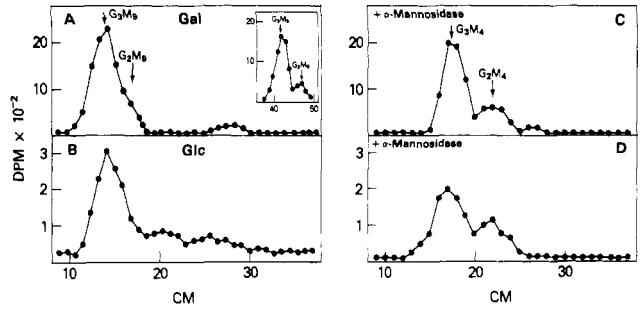

FIGURE 3: Labeled lipid-released oligosaccharides from tumor minces incubated for 60 min in the presence of [^{3}H]galactose or [^{3}H]glucose. Oligosaccharides derived from [^{3}H]galactose labeling (A) or [^{3}H]-glucose labeling (B) were subjected to paper chromatography for 24 or 48 h (panel A inset). After α-mannosidase treatment, the oligosaccharides initially derived from [^{3}H]galactose labeling (C) or [^{3}H]glucose labeling (D) were chromatographed for 16 h. The standards are as described in the legend to Figure 2.

and further treated with α-mannosidase (Figure 2B,C). Peak I was only partially digested into a major and minor species (Figure 2B), whereas peaks II, III, and IV were largely degraded to free labeled mannose (Figure 2C). The migration of the two partially resistant oligosaccharides in peak I was identical with that of $Glc_3Man_4GlcNAc_2$ and $Glc_2Man_4GlcNAc_2$, respectively. This finding suggests that peak I was composed of two mannose-rich oligosaccharides varying in the number of blocking groups, presumably glucose residues. The same array of oligosaccharide lipids was observed at 10, 30, or 120 min of incubation with mannose (data not shown). After 60 min of labeling, the average amount of radioactivity was 40% in peak I, 45% in peak II plus III, and 15% in peak IV.

The presence of glucose in the largest oligosaccharides was demonstrated more directly by incubating tumor minces for 60 min in presence of [^{3}H]galactose or [^{3}H]glucose (Figure 3). As previously reported (Chapman et al., 1979; Hubbard & Robbins, 1979), galactose is readily converted to UDP-galactose and then to UDP-glucose within the cell. In this experiment, the only species labeled with both isotopes were of large molecular weight (Figure 3A,B). A longer migration showed that the main peak was composed of two oligosaccharides migrating as $Glc_3Man_9GlcNAc_2$ and $Glc_2Man_9GlcNAc_2$ (Figure 3A, inset). Accordingly, two partially resistant oligosaccharides were detected after α-mannosidase, again comigrating with $Glc_3Man_4GlcNAc_2$ and $Glc_2Man_4GlcNAc_2$ (Figure 3C,D). One hundred percent of the initial galactose labeling and approximately 85% of the initial glucose labeling were recovered in the latter oligosaccharides, showing that as already found by others (Chapman et al., 1979; Hubbard & Robbins, 1979) galactose labeling provides a better marker of glucose residues than does glucose labeling, since the latter sugar is to some extent converted to mannose (see Figure 4F).

Thus, in mouse thyrotropic tumors, oligosaccharide lipids were of different sizes, ranging from $Man_5GlcNAc_2$ to $Glc_3Man_9GlcNAc_2$ as reported for other tissues such as CHO cells (Chapman et al., 1979), fibroblasts (Hubbard & Robbins, 1979), or thyroid (Ronin et al., 1981; Godelaine et al., 1981). Recently we have found that the conditions of incubation may affect the relative amounts of such forms, with more $Glc_3Man_9GlcNAc_2$ noted in minces with an atmosphere of 95% oxygen or in dispersed cells under various conditions (data not shown).

Characterization of Oligosaccharides Derived from Total Cell Glycoproteins. To analyze the size of the protein-linked

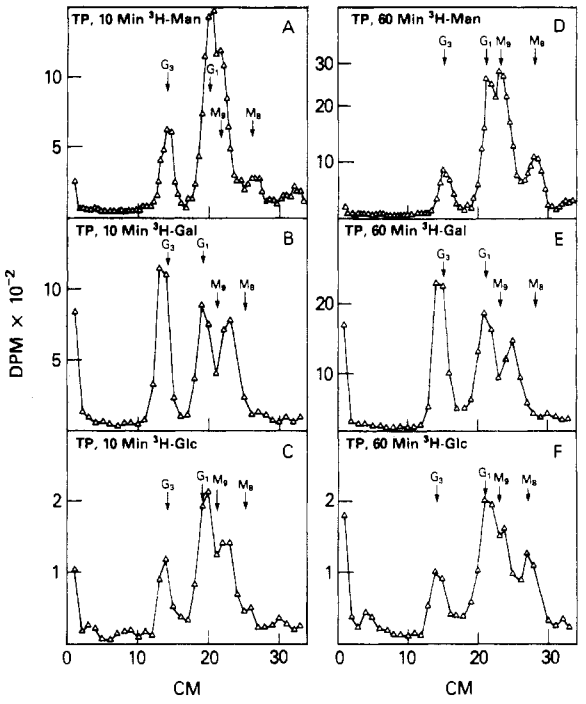

FIGURE 4: Paper chromatography of oligosaccharides from total cell proteins after endo H treatment. Tumor minces were labeled for 10 min with [^{3}H]mannose (A), [^{3}H]galactose (B), or [^{3}H]glucose (C) or for 60 min with the same three isotopes (panels D, E, and F, respectively). The chromatogram was developed for 48 h. The standards are $Glc_3Man_9GlcNAc$ (G_3), $Glc_1Man_9GlcNAc$ (G_1), $Man_9GlcNAc$ (M_9), and $Man_8GlcNAc$ (M_8). Radioactivity at the origin represents endo H resistant oligosaccharides not released from tryptic glycopeptides.

oligosaccharides, tumor minces were labeled for 10 or 60 min in presence of [^{3}H]mannose, [^{3}H]galactose, or [^{3}H]glucose. As shown in Figure 4, nearly all of the labeled material could be released from tryptic glycopeptides by endo H after either 10 or 60 min of labeling. Several oligosaccharides were identified, all migrating slower than a $Man_7GlcNAc$ standard. With mannose labeling, labeled oligosaccharides were found migrating to the position of $Glc_3Man_9GlcNAc$, $Glc_1Man_9GlcNAc$, $Man_9GlcNAc$, and $Man_8GlcNAc$ standards (Figure 4A). As the pulse time increased to 60 min, a slight progression was observed from $Glc_3Man_9GlcNAc$ and $Glc_1Man_9GlcNAc$ to $Man_9GlcNAc$ and $Man_8GlcNAc$ (compare panels D and A of Figure 4). Treatment of these oligosaccharides with α-mannosidase generated two partially resistant oligosaccharides comigrating with $Glc_3Man_4GlcNAc$ and $Glc_1Man_4GlcNAc$, suggesting that among them at least two differed in their glucose content (data not shown). Labeling tumor minces with [^{3}H]galactose revealed three glucose-containing oligosaccharides, two comigrating with $Glc_3Man_9GlcNAc$ and $Glc_1Man_9GlcNAc$, respectively, and one migrating between $Man_9GlcNAc$ and $Man_8GlcNAc$ (Figure 4B,E). Such an oligosaccharide was suggested to be $Glc_1Man_8GlcNAc$ by Parodi & Cazzulo (1982) using the same chromatographic system as well as by others (Kornfeld et al., 1978; Godelaine et al., 1978). Similar data were obtained with glucose labeling (Figure 4C,F), although some interconversion to mannose probably occurred at 60 min with the appearance of a labeled $Man_9GlcNAc$ species (Figure 4F). Therefore, glucose labeling was no longer used to specifically identify glucose in subsequent experiments.

Characterization of TSH Subunit Oligosaccharides. Previous studies using [^{35}S]methionine have shown that intracellular TSH α and β subunits are largely endo H sensitive after 60 min of continuous labeling and even up to 18 h (Magner & Weintraub, 1982; Weintraub et al., 1983). TSH and free α subunit are secreted into the medium after approximately 2 h of incubation, and the oligosaccharides are at that time virtually all endo H resistant (Weintraub et al., 1983). Thus, to elucidate the early processing events occurring on TSH carbohydrate chains, tumor minces were labeled for 10 min with [^{3}H]mannose or [^{3}H]galactose then washed and chased for 60 and 120 min (Figure 5). The mannose-labeled oligosaccharides present on both α and β subunits were less heterogeneous than those bound to total proteins: after a 10-min pulse, only one species comigrating with $Man_9GlcNAc$ was observed either on TSH + α (Figure 5A) or on free β (Figure 5D) in contrast to the four usual oligosaccharides present on total proteins (Figure 5G). At the same time, no glucose could be detected by using galactose labeling on both TSH subunits (Figure 5A,D), while $Glc_3Man_9GlcNAc$, $Glc_1Man_9GlcNAc$, and $Glc_1Man_8GlcNac$ were clearly labeled on total proteins (Figure 5G). After 60 min of chase, a new mannose-labeled species comigrating with $Man_8GlcNAc$ was detected on TSH + α (Figure 5B) and on free β subunits (Figure 5E), and also in increasing amount on total proteins (Figure 5H). Again no glucose was found on TSH subunits while the three glucose-containing oligosaccharides were still detected on total proteins (compare panels B and E to panel H of Figure 5). Extending the chase to 120 min showed the accumulation of $Man_8GlcNAc$ on TSH + α (Figure 5C) while $Man_9GlcNAc$ was still the predominant species on free β (Figure 5F). The array of oligosaccharides released from total proteins at that time was highly heterogeneous, exhibiting still some $Glc_3Man_9GlcNAc$ together with other species as small as $Man_6GlcNAc$ (Figure 5I). The two largest species of Glc_3 and Glc_1 were always labeled on total proteins whereas they were undetectable on TSH subunits.

Since no $Glc_3Man_9GlcNAc_2$ was found on TSH subunits in these pulse–chase experiments, attempts were made to reduce the time of labeling. Reducing the pulses to 2 or 5 min either with [^{3}H]mannose or with [^{3}H]galactose yielded data similar to those of a 10-min pulse (data not shown), although the low level of radioactivity precipitated under such conditions precluded identification of small percentages of $Glc_3Man_9GlcNAc_2$. Therefore, carbohydrate processing rates appeared clearly different on TSH compared to total proteins. Moreover, although the initial glycosylation of the two TSH subunits appeared very similar, their carbohydrate processing proceeded at different rates.

Control Experiments. Since TSH subunits were isolated through selective immunoprecipitations, we first investigated whether glucose could have been artifactually removed during this long, multistep, nondenaturing procedure. To assess putative glucosidase activity in the 100000*g* supernatant of the detergent extract or in the antisera, aliquots were immunoprecipitated with anti-α either for 18 h at 4 °C or by the standard method of 2 h at 37 °C followed by 16 h at 4 °C. Identical profiles of mannose-labeled oligosaccharides released from α subunits were found in both cases (data not shown). Moreover, when the supernatants remaining after anti-α precipitation at either 4 or 37 °C were precipitated with trichloroacetic acid, the pattern of oligosaccharides on total proteins was identical with that observed with acid precipitation alone. This indicates that no degradation of oligosaccharides had occurred either for TSH or for total proteins during the usual immunoprecipitation procedure at 37 °C. Moreover, in rat liver, the membranous glucosidases were shown to be inactivated by Tris buffer (Ugalde et al., 1979).

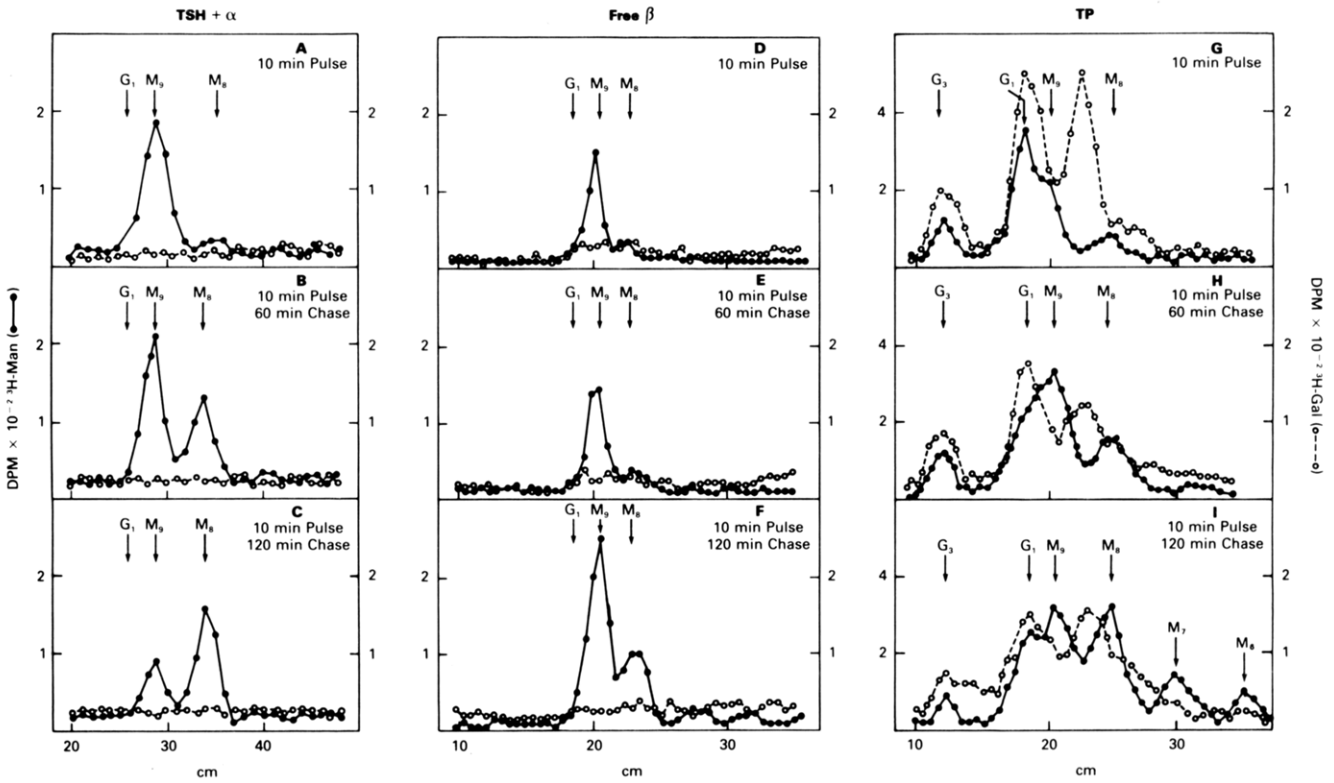

FIGURE 5: Paper chromatography of endo H released oligosaccharides from TSH + α (left panels), free β (middle panels), and total proteins (right panels). Tumor minces were pulsed for 10 min (A, D, G) with [^{3}H]mannose or [^{3}H]galactose and chased for either 60 min (B, E, H) or 120 min (C, F, I). The chromatogram was developed for 48 h. The standards are as described in the legend to Figure 4 plus $Man_7GlcNAc$ (M_7) and $Man_6GlcNAc$ (M_6).

To exclude a possible contamination of total proteins with oligosaccharide lipids, the trichloroacetic acid precipitates were boiled for 5 min before being washed, since this method has previously been shown to fully release oligosaccharides from their lipid carriers (Ronin et al., 1978). We found no modification of total protein carbohydrate chains after such treatment (data not shown). We also compared the trichloroacetic acid method of isolating total proteins to our sequential delipidation method (Ronin & Bouchilloux, 1976, 1978) and, again, found no major differences (data not shown). Therefore, none of the oligosaccharides prepared from total cell glycoproteins was originally derived from lipid carriers.

Finally, TSH subunits were isolated without immunoprecipitation by using entirely chemical procedures under completely denaturing conditions. Tumor minces were incubated for 60 min in the presence of [^{3}H]mannose or [^{3}H]galactose, and the incubation was terminated by adding chloroform and methanol. Following delipidation, total proteins were analyzed by SDS gel electrophoresis under denaturing conditions involving boiling and reduction. As shown in Figure 6, mannose labeling was mainly concentrated in the 22K and 18K regions previously demonstrated to be composed of TSH α and β subunits, respectively (Weintraub et al., 1980). In contrast, galactose labeling was more widely spread among proteins, especially those of high molecular weight. Proteins from four different areas of the gel were selected, eluted, precipitated with 8 volumes of acetone at −20 °C for 24 h, and subsequently treated with trypsin and endo H. In the larger molecular weight regions of the gel, most of the radioactivity derived from the galactose labeling was found to be associated with endo H resistant oligosaccharides (Figure 7A,B), whereas in the TSH region, the major part of the label was composed of endo H sensitive oligosaccharides (Figure 7C,D). Previous studies have shown that most of these higher molecular weight non-TSH glycoproteins are nonsecretory (Weintraub et al., 1983)

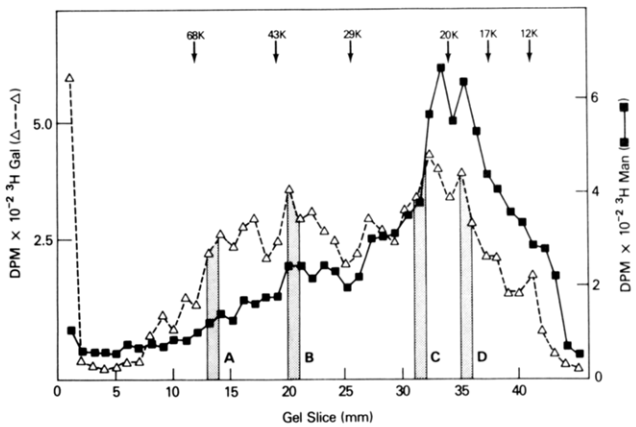

FIGURE 6: SDS–polyacrylamide gel electrophoresis of delipidated total proteins labeled for 60 min with either [^{3}H]mannose or [^{3}H]galactose. Different areas of the gel corresponding to either unknown proteins (A and B) or TSH-α (C) and TSH-β (D) were extracted for oligosaccharide characterization.

and are presumably membranous. Although $Glc_3Man_9GlcNAc_2$ was still detectable on total cell glycoproteins at this time (Figure 4) and on selected nonsecretory proteins (Figure 7A), it was not seen on the 22K α-protein (Figure 7C) or on the 18K β-protein (Figure 7D). The labeled oligosaccharides instead comigrated with $Glc_1Man_9GlcNAc$ and $Glc_1Man_8GlcNAc$ on both α (Figure 7C) and β (Figure 7D) subunits. Even if there were any contamination of the TSH subunit region by some unknown glycoprotein, the results showed again that no $Glc_3Man_9GlcNAc_2$ was present on TSH carbohydrate chains in contrast to certain other nonsecretory cell glycoproteins. Moreover, the isolation of TSH subunits by chemical means also rules out the possibility that our antisera did not recognize unfolded conformations of the subunits containing $Glc_3Man_9GlcNAc_2$.

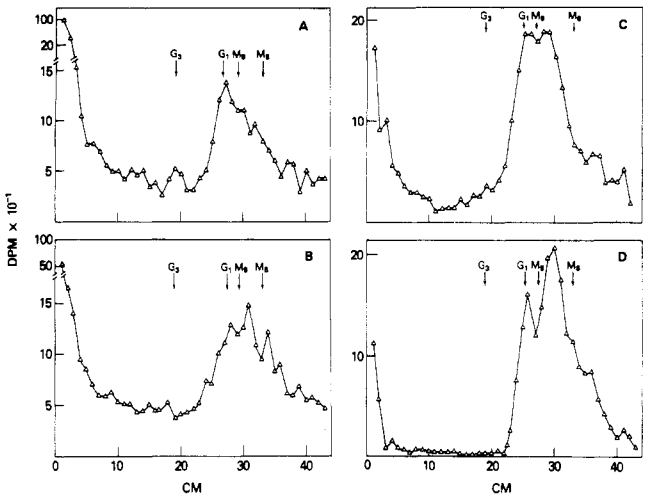

FIGURE 7: Paper chromatography of [^{3}H]galactose-labeled oligosaccharides from proteins A, B, C, and D isolated as described in Figure 6 and treated with endo H. The standards are as described in the legend to Figure 4, and the chromatogram was developed for 48 h.

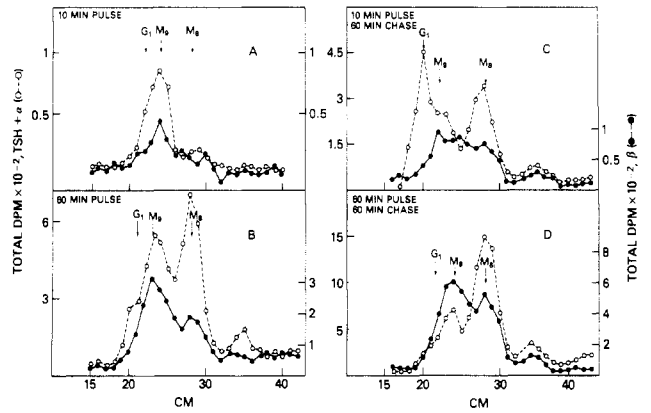

FIGURE 8: Paper chromatography of oligosaccharides from TSH + α and from free β labeled for various times with [^{3}H]mannose in the presence of 5% nondialyzed hypothyroid calf serum. Tumor minces were pulsed for 10 (A) or 60 min (B) with [^{3}H]mannose and chased for 60 min (panels C and D, respectively). The standards are as described in the legend to Figure 4, and the chromatogram was developed for 48 h.

Modification of TSH α-Glycosylation in the Presence of Serum. In certain instances, tumor minces were incubated in the presence of hypothyroid calf serum. Figure 8 shows the endo H released oligosaccharides from TSH + α and from free β subunits after an incubation of 10 or 60 min in the presence of [^{3}H]mannose and 5% nondialyzed hypothyroid calf serum followed by a chase of 60 min. The 10-min pulse showed again primarily the presence of $Man_9GlcNAc$ on α and β subunits (Figure 8A) whereas a 60-min chase resulted in the predominance of a species comigrating with $Glc_1Man_9GlcNAc$ on TSH + α but not free β (Figure 8C). This species must be transient since it is barely detectable in a 60-min pulse (Figure 8B) as well as in a 60-min pulse followed by a 60-min chase (Figure 8D). At both times, $Man_8GlcNAc$ appeared to accumulate on TSH + α whereas this form was less prominent on free β subunits (Figure 8B–D). At all time points, total protein carbohydrate chains appeared unmodified by the presence of serum (data not shown), indicating that the modification of glycosylation was largely restricted to TSH subunits.

The presence of a species comigrating with $Glc_1Man_9GlcNAc_2$ on TSH + α subunits was also detected when dispersed cells were used instead of minces[2] probably because enzymatic dispersion of the tumor tissue (Weintraub et al., 1983) was performed in the presence of hypothyroid calf serum. This suggests that the presence of glucose on TSH or α subunits may depend on the stimulation of thyrotropic cells by unknown factors present in hypothyroid serum.

Discussion

In this study, we have identified various stages on TSH subunit glycosylation and compared them to total cell protein glycosylation. In thyrotropic tumors, most of the large molecular weight cellular glycoproteins have been previously identified as nonsecretory, presumably membrane, proteins (Weintraub et al., 1983). Differences were found in the processing of the two types of glycoproteins, indicating that the biosynthesis of the carbohydrate chains is closely dependent on the nature of the polypeptide backbone.

Like those in a variety of other mammalian tissues (Hubbard & Ivatt, 1981), lipid-linked oligosaccharides from thyrotropic tumors are composed of large molecular weight oligosaccharides containing glucose residues. This is consistent with previous reports on the presence of dolichol in beef pituitary gland (Caroll et al., 1973) and the cell-free labeling of glucose-containing oligosaccharide lipids in calf pituitary gland (Henner et al., 1981). Although $Glc_3Man_9GlcNAc_2$ was always a major component on lipid-linked oligosaccharides from thyrotropic tumors, its relative amount varied among experiments, and under certain conditions, the predominant species was $Man_9GlcNAc_2$.

$Glc_3Man_9GlcNAc_2$ was consistently found on total tumor proteins, strongly supporting the idea that glycosylation of the majority of cell nonsecretory glycoproteins does occur by transfer of this species from the lipid carrier. In chick embryo fibroblasts, the half-life of $Glc_3Man_9GlcNAc_2$ on proteins was less than 2 min (Hubbard & Robbins, 1979), and in calf thyroid, it appeared to be close to 15 min (Godelaine et al., 1981), whereas in the present study it was surprisingly slower, approximately 2 h. We have also observed similar data with normal rat pituitaries,[2] indicating that this prolonged half-life is not related to the tumoral process. Moreover, within the tumor, selected nonsecretory glycoproteins isolated from SDS gels proved to be trimmed of their glucose content at different rates, suggesting that glucosidase action could differ from one protein to another. As found by others (Kornfeld et al., 1978; Hubbard & Robbins, 1979), two glucose residues are rapidly removed, whereas the Glc_1 intermediate seemed to be more stable. Pulse–chase experiments have indicated that this species might be a key intermediate with respect to the removal of the first mannose residue: either glucose is removed before mannose trimming begins, resulting in the Man_9 intermediate, or it is still present when the first mannose residue is excised, resulting in Glc_1Man_8. Both pathways are likely to occur in the pituitary, and how they relate to the synthesis of high-mannose vs. complex oligosaccharides remains to be elucidated.

A striking feature of the present study is that in contrast to total proteins, no $Glc_3Man_9GlcNAc_2$ was detected on either TSH subunit under various labeling conditions and using different isolation procedures. Instead, $Man_9GlcNAc_2$, and under certain conditions a species comigrating with $Glc_1Man_9GlcNAc2$, was the largest species found on α and β subunits. It is unlikely that the putative $Glc_1Man_9GlcNAc_2$ form arises from direct transfer from the lipid carrier since this species was usually absent or was a minor component of oligosaccharide lipids labeled with either mannose or galactose. The other possibilities for the presence of $Glc_1Man_9GlcNAc_2$ on TSH are either that TSH glycosylation occurs through a Glc_3-containing precursor which is then very rapidly trimmed

or that it occurs by transfer of a $Man_9GlcNAc_2$ precursor followed by the addition of glucose. Alternatively, this species may be another endo H sensitive oligosaccharide that comigrates with Glc_1Man_9, and more definitive structural studies will be required to clarify this point.

Control experiments were designed toward the elucidation of possible preferential processing of TSH carbohydrate chains compared to those of cell glycoproteins. Reducing the time of labeling to 2 min, the shortest time yielding detectable radioactivity in the immunoprecipitates, did not show a $Glc_3Man_9GlcNAc_2$ form as it did for total proteins of this thyrotropic tumor as well as those of chick embryo fibroblasts (Hubbard & Robbins, 1979). In the latter study, only approximately 30% of the radioactivity was released by endo H as $Glc_3Man_9GlcNAc_2$, raising the possibility that it might not be the unique precursor that is transferred. Our second approach was to purify TSH subunits by chemical means only, thereby preventing any possible enzymatic degradation or selective isolation by antisera. Using delipidation starting at the end of the incubation followed by gel electrophoresis analysis, we again failed to detect any Glc_3 species as had been found in the case of thyroglobulin (Godelaine et al., 1981). However, since thyroglobulin is known to contain both high-mannose and complex-type oligosaccharides (Tsuji et al., 1981; Yamamoto et al., 1981), it remains unclear whether this precursor form could be restricted to only one type of carbohydrate moiety.

$Glc_1Man_9GlcNAc_2$ appeared to be an important intermediate in TSH processing. In certain pulse–chase experiments using serum, this form could not be detected on TSH + α during the pulse but was present during the chase. This species has also been found to be modulated on β subunits by various hormones.[2] Finally, preliminary results[3] have shown that this intermediate was present in higher amount in the smooth endoplasmic reticulum than in the rough membranes. We have previously reported the accumulation of this intermediate on thyroid proteins (Ronin & Caseti, 1981), and similar data have been reported for *Trypanosoma cruzi* (Parodi & Cazzulo, 1982).

Another rate-limiting step in TSH subunit processing appeared to be the $Man_8GlcNAc_2$ intermediate. This species is predominant with time on α subunits while present in lesser amount on β subunits. This form was also reported to accumulate on the human CG α subunit (Ruddon et al., 1981) and on yeast glycoproteins (Byrd et al., 1982). The relationship of carbohydrate processing to TSH subunit combination could not be deduced from the present experiments because the immunoprecipitation methods generally used here did not distinguish between free and combined subunits, and also because at most of the early labeling times chosen for the present study, α or β subunits are largely uncombined (Magner & Weintraub, 1982). However, other work has demonstrated that once combined, the subunits are readily converted to endo H resistant, presumably complex-type structures (Weintraub et al., 1983).

Finally, the present work provides evidence that the processing of certain secretory glycoproteins is different from that of certain nonsecretory glycoproteins in the same cell. Both the rate and the individual steps of this mechanism appear to be specifically modulated. Similarly, in hepatoma cells, it was found that the VSV protein G, a membrane protein, requires 20 min before reaching the cell surface, while transferrin, a secretory protein, needs more than 1 h (Strous & Lodish, 1980) although no structural studies have been performed with oligosaccharides from these proteins. Since the two types of proteins follow different intercellular routes, it is possible that glucose and mannose removal might function as sorting signals in this pathway. For example, the presence of glucose might protect the carbohydrate chain from mannosidase action in specific cellular locations or might designate certain of the mannoses to be trimmed. Alternatively, glucosylated oligosaccharides might interact with specific membrane receptors as has been recently proposed by Parodi et al. (1983). In fact, in Con A resistant mutant CHO cells, deficient in the glucosylation of oligosaccharide lipids, the carbohydrate chains of lysosomal enzymes are finally converted to complex-type instead of high-mannose-type structures and are not sequestered in the normal lysosomal compartment (Krag & Robbins, 1982). In this case, it has been clearly established that mannose 6-phosphate is the usual sorting signal which is not formed in the mutant line deficient in glucosylation.

Recently Spiro et al. (1983) have demonstrated that energy deprivation in thyroid slices could modify glucosylation of oligosaccharide lipids and decrease glycosylation of proteins. Although the current studies were performed with minces in an atmosphere of 95% air–5% CO_2, we have also performed similar studies with minces in an atmosphere of 95% O_2–5% CO_2 as well as with enzymatically dispersed cells under various conditions of oxygenation and glucose and serum concentration (data not shown). Under all these incubation conditions, the processing of TSH subunit and total protein oligosaccharides was similar to that reported herein. In agreement with Spiro et al. (1983), we did find that high oxygenation of minces or cells increased the relative proportion of $Glc_3Man_9GlcNAc_2$ on lipid oligosaccharides. Nonetheless, it will be important to study further the effects of various incubation conditions on the regulation of TSH subunit and total glycoprotein carbohydrate processing.

Registry No. TSH, 9002-71-5.

[3] See Magner et al. (1983).

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The Mammalian β_2-Adrenergic Receptor: Purification and Characterization†

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ABSTRACT: The β_2-adrenergic receptors from hamster, guinea pig, and rat lungs have been solubilized with digitonin and purified by sequential Sepharose–alprenolol affinity and high-performance steric-exclusion liquid chromatography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and autoradiography of iodinated purified receptor preparations reveal a peptide with an apparent M_r of 64 000 in all three systems that coincides with the peptide labeled by the specific β-adrenergic photoaffinity probe (*p*-azido-*m*-[^{125}I]iodobenzyl)carazolol. A single polypeptide was observed in all three systems, suggesting that lower molecular weight peptides identified previously by affinity labeling or purification in mammalian systems may represent proteolyzed forms of the receptor. Purification of the β-adrenergic receptor has also been assessed by silver staining, iodinated lectin binding, and measurement of the specific activity (~15 000 pmol of [^{3}H]dihydroalprenolol bound/mg of protein). Overall yields approximate 10% of the initial crude particulate binding, with 1–3 pmol of purified receptor obtained/g of tissue. The purified receptor preparations bind agonist and antagonist ligands with the expected β_2-adrenergic specificity and stereoselectivity. Peptide mapping and lectin binding studies of the hamster, guinea pig, and rat lung β_2-adrenergic receptors reveal significant similarities suggestive of evolutionary homology.

Previous studies of the β-adrenergic receptor from this laboratory have documented development of procedures for solubilization (Caron & Lefkowitz, 1976), affinity chromatography (Caron et al., 1979), and total purification of the receptor from both amphibian (Shorr et al., 1981, 1982a) and avian erythrocytes (Shorr et al., 1982b). While these sources have proven to be valuable model systems for study of the β-adrenergic receptor, the purification and characterization of mammalian β-adrenergic receptors is of potentially greater interest. Recently, the purification of the β_2-adrenergic receptor from canine lung has been described (Homcy et al., 1983). However, as judged by sodium dodecyl sulfate (SDS) gel electrophoresis, the polypeptide isolated by Homcy et al. (1983) was apparently smaller (i.e., M_r = 52 000–53 000) than that of the β_2-adrenergic receptor in several other mammalian systems as determined by photoaffinity labeling in membrane preparations (Lavin et al., 1982; Benovic et al., 1983; Stiles

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