Glucosylation of Glycoproteins by Mammalian, Plant, Fungal, and Trypanosomatid Protozoa Microsomal Membranes†

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Received April 6, 1989; Revised Manuscript Received June 8, 1989

ABSTRACT: An assay for UDP-Glc:glycoprotein glucosyltransferase was developed. Incubation of rat liver microsomes with UDP-[^{14}C]Glc led to the formation of hot trichloroacetic acid insoluble material identified as protein-linked $Glc_1Man_{7-9}GlcNAc_2$. Addition of 8 M urea-denatured thyroglobulin to the incubation mixtures stimulated up to 10–12-fold the formation of the same compounds but only in the presence of detergents. Native thyroglobulin was ineffective. Several experiments indicated that the stimulation was due to the transfer of glucose residues from UDP-Glc to high-mannose oligosaccharides in urea-denatured thyroglobulin and that this transfer reaction did not involve dolichol mono- or diphosphate derivatives as intermediates. The glycoprotein glucosylating activity was mainly located in the endoplasmic reticulum and could glucosylate glycopeptides derived from the digestion of thyroglobulin with an unspecific protease. Glucosylation of oligosaccharides in those glycopeptides occurred, however, at a rate at least 2 orders of magnitude slower than that of the same compounds in urea-denatured thyroglobulin. Tryptic digestion of urea-denatured thyroglobulin did not affect its glucosylation rate. The structure of $Glc_1Man_9GlcNAc_2$ linked to urea-denatured thyroglobulin was identical with that of $Glc_1Man_9GlcNAc_2$-P-P-dolichol. The assay of UDP-Glc:glycoprotein glucosyltransferase allowed detection of the activity in microsomal membranes in which endogenous acceptors appeared to be absent or almost absent, such as those derived from mung bean, *Mucor rouxii*, *Crithidia fasciculata*, and *Trypanosoma cruzi* cells. These results provide evidence for the transfer of glucose residues directly from UDP-Glc to high-mannose oligosaccharides in glycoproteins in the endoplasmic reticulum of mammalian, plant, fungal, and protozoan cells and indicate that the previously characterized glucosidase II probably is responsible for the processing of glucosylated glycoproteins.

In most eucaryotes N-glycosylation is initiated by the transfer of $Glc_3Man_9GlcNAc_2$ from a dolichol-P-P derivative to asparagine residues in incompleted polypeptide chains in the lumen of the rough endoplasmic reticulum (Kornfeld & Kornfeld, 1985). Processing of the oligosaccharide is initiated immediately after being transferred: the three glucose units are removed by two specific glucosidases located in the endoplasmic reticulum, glucosidase I that excises the external $\alpha(1-2)$-linked unit and glucosidase II that removes both $\alpha(1-3)$-linked residues (Kornfeld & Kornfeld, 1985). In addition, some mannoses may be then removed by two mannosidases also located in the endoplasmic reticulum, one sensitive and the other insensitive to the inhibitor deoxymannojirimycin (Bischoff & Kornfeld, 1983; Rizzolo & Kornfeld, 1988).

Trypanosomatid protozoa are unique in nature as they transfer to protein unglucosylated oligosaccharides $Man_{6,7,9}GlcNAc_2$, depending on the species (Parodi & Quesada-Allue, 1982; Parodi et al., 1981, 1984a; Previato et al., 1986). They are unable to synthesize dolichol-P-Glc, the donor of the glucose units in the complete oligosaccharide in mammalian, avian, fungal, and plant cells (de la Canal & Parodi, 1987). Notwithstanding the fact that the unglucosylated oligosaccharides are transferred to proteins in those microorganisms, transiently glucosylated, protein-linked high-mannose oligosaccharides were detected in cells incubated with [^{14}C]glucose ($Glc_1Man_{5-9}GlcNAc_2$, depending on the species) (Mendelzon & Parodi, 1986; Mendelzon et al., 1986; Parodi & Cazzulo, 1982; Parodi et al., 1983a, 1984a). The transient formation of $Glc_1Man_{7-9}GlcNAc_2$–protein was also detected in mammalian cells incubated with the same labeled precursor (Parodi et al., 1983b; 1984b). In all cell types the glucosylated compounds disappeared upon chasing incubations with unlabeled glucose. Evidence obtained in in vitro assays showed that in calf thyroid cells protein-linked $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ were produced by glucosylation of $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ (pathway B, Figure 1) and not by demannosylation of $Glc_1Man_9GlcNAc_2$ (pathway A, Figure 1): upon incubation of cells with [^{14}C]glucose, label in the glucose residues of protein-bound $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, and $Glc_1Man_7GlcNAc_2$ appeared almost instantly, whereas there was a relatively long delay in the appearance of label in the mannose units of $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ once it had appeared in the same residues of $Glc_1Man_9GlcNAc_2$. The time interval required for labeling the mannose and glucose residues in the three glucosylated compounds should have been similar if formation of protein-linked $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ had taken place according to pathway A. On the other hand, the observed difference in the labeling interval is compatible with pathway B. In addition, label in the mannose units of $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ appeared only in samples in which the incubation time had been sufficient for $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ labeling (Parodi et al., 1983b). A similar analysis performed with *Trypanosoma cruzi* cells incubated with [^{14}C]glucose showed that protein-linked $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ were produced by glucosylation of $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ and not by demannosylation of $Glc_1Man_9GlcNAc_2$ (Parodi et al., 1983a).

Evidence obtained from in vitro assays performed with calf

† This work was supported by grants from the National Research Council (Argentina), from the University of Buenos Aires, and from the United Nations Development Program/World Bank/World Health Organization Special Program for Research and Training in Tropical Diseases.

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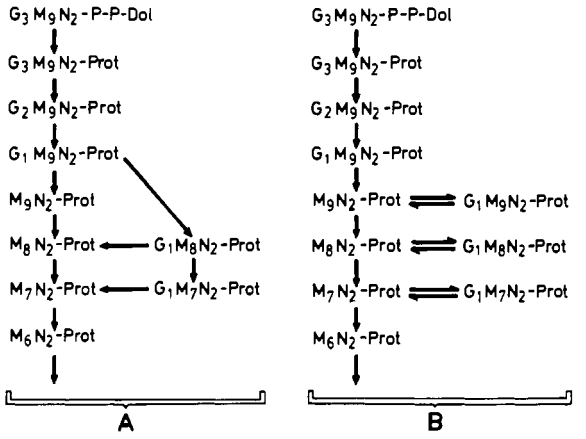

FIGURE 1: Alternative pathways of oligosaccharide processing. Note that according to pathway A formation of glucose-labeled $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, and $Glc_1Man_7GlcNAc_2$ necessarily involves first synthesis of dolichol-P-Glc, then of $Glc_3Man_9GlcNAc_2$-P-P-dolichol, followed by transfer of the oligosaccharide to Asn residues in proteins and deglucosylation and demannosylation of the compound. According to pathway B, glucose labeling of the above-mentioned saccharides may occur by direct transfer of glucose units from UDP-Glc to $Man_{7-9}GlcNAc_2$. G, M, N, Dol, and Prot represent glucose, mannose, *N*-acetylglucosamine, dolichol, and protein, respectively.

thyroid and rat liver membranes showed not only that protein-linked $Glc_1Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ were produced by glucosylation of $Man_8GlcNAc_2$ and $Glc_1Man_7GlcNAc_2$ but also that at least part of $Glc_1Man_9GlcNAc_2$ was formed by a similar mechanism (pathway B, Figure 1) (Parodi et al., 1984b). All in vitro assays were performed by using endogenous glycoproteins of rat liver and calf thyroid membranes as acceptors for glucosylation. This approach had several limitations as dependence on endogenous acceptors precluded characterization of the glycoprotein domain recognized by the glucose-transferring activity, purification, and further characterization of the enzyme as well as detection of its true subcellular distribution. To overcome these shortcomings, we have developed an in vitro assay for the glucosylation of glycoproteins using as exogenous acceptor denatured thyroglobulin, a glycoprotein having high-mannose oligosaccharides. The assay allowed us to detect the glucose-transferring activity not only in mammalian systems but also in membranes devoid or almost devoid of endogenous acceptors as those derived from plant, fungal, and protozoan cells. In some of them no evidence for the transient glucosylation of glycoprotein was available.

Materials and Methods

Materials, Substrates, and Standards. Jack bean α-mannosidase, bovine thyroglobulin, *Streptomyces griseus* protease type XIV, endo-β-*N*-acetylglucosaminidase H, 1-deoxynojirimycin, trypsin, and soybean trypsin inhibitor were from Sigma (St. Louis, MO). The following glucosyl–glucose disaccharides were also purchased from Sigma: nigerose, α(1,3); maltose, α(1,4); isomaltose, α(1,6); laminaribiose, β(1,3); cellobiose, β(1,4); and gentiobiose, β(1,6). Kojibiose, α(1,2), and sophorose, β(1,2), were purchased from Koch-Light, Buckinghamshire, U.K. Amphomycin was a gift from Dr. Alan Elbein, University of Texas, San Antonio, TX. UDP-[^{14}C]Glc (285 Ci/M) was prepared according to the method of Wright and Robbins (1965) with minor modifications.

[*glucose*-^{14}C]$Glc_{1-3}Man_9GlcNAc_2$-P-P-dolichol was prepared by incubating UDP-[^{14}C]Glc with rat liver microsomes as described previously (Parodi et al., 1973). Mild acid hydrolysis of these compounds followed by treatment with endo-β-*N*-acetylglucosaminidase H produced the standards $Glc_{1-3}Man_9GlcNAc$. Standards [^{14}C]$Man_{7-9}GlcNAc$ were obtained from hen oviduct slices incubated with [^{14}C]glucose as previously described (Parodi et al., 1981). The hexapeptide Tyr-Asn-Leu-Thr-Ser-Val was generously synthesized by Drs. Ulf Hellman and Ulla Engstrom (Ludwig Institute for Cancer Research—Uppsala Branch, Uppsala, Sweden). It had its C-terminal end amidated (Bosch et al., 1988). High-mannose glycopeptides were prepared as follows: 100 mg of thyroglobulin was incubated in 3.5 mL of 0.125 M Tris-HCl buffer, pH 8.0, 3.5 mM $CaCl_2$, and 3 mg of *S. griseus* protease for 4 days at 37 °C under a toluene atmosphere. Another milligram of protease was added after 24 h of incubation. The solution was then heated for 5 min at 100 °C and centrifuged, and the supernatant was applied to a 0.5 × 7 cm column of concanavalin A–Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with 10 mM Tris-HCl buffer, pH 8.0, 0.15 M NaCl, 1 mM $MgCl_2$, and 1 mM $CaCl_2$. The column was washed first with 21 mL of the last solution and then with 14 mL of 10 mM methyl α-glucoside in the same solution. Glycopeptides were then eluted with 21 mL of 0.1 M methyl α-mannoside in the same solution. The solution containing glycopeptides was divided into halves; each half was concentrated to 2 mL and applied to a 1.2 × 55 cm Sephadex G-10 column equilibrated with 7% 2-propanol. Glycopeptides eluted in the void volume. They were dried and dissolved in water at a concentration equivalent to 0.5 mg of mannose/mL.

Loss of high-mannose glycopeptides during their purification (20–30%) was monitored with [*glucose*-^{14}C]-$Glc_1Man_{7-9}GlcNAc_2$–peptide, derived from the proteolytic treatment of glucosylated thyroglobulin, as internal standards. Total amount of high-mannose oligosaccharides in thyroglobulin appeared to be 1.5–2.0%, a value in good agreement with that reported by Arima et al. (1972).

Membrane Preparations. Rat liver microsomes were prepared as described previously (Parodi et al., 1972). *Crithidia fasciculata* and *T. cruzi* cells were grown, and membranes from them were prepared as before (de la Canal & Parodi, 1987). Mycelial-form cells of *Mucor rouxii*, strain NRRL 1894, were grown, and membranes from them were prepared as described before for *Saccharomyces cerevisiae* (Parodi, 1979). Mung bean sprouts (200 g) bought at the local market were disintegrated in 300 mL of 0.25 M sucrose, 5 mM NaEDTA, pH 7.7, and 5 mM 2-mercaptoethanol in an Omnimixer blender and passed through cheesecloth. The suspension was then centrifuged at 19800*g* for 15 min and the supernatant centrifuged for 60 min at 100000*g*. The pellet was then resuspended in the same solution at protein concentrations of 25–30 mg/mL. Rat liver, *C. fasciculata*, and *M. rouxii* membranes were also finally resuspended in the same solution at approximately the same protein concentration. The method used for obtaining membranes from the rough and smooth endoplasmic reticula and from the Golgi apparatus of rat liver was the same as that described before (Parodi et al., 1984b) under the title "Rough, Smooth Golgi-free and Crude membranes" with the following modifications: (a) the membranes appearing in the 0.5–1 M sucrose interphase of the gradient centrifugation of the smooth membranes were not discarded but taken as Golgi membranes; (b) the rough, smooth, and Golgi membranes appearing after a first gradient centrifugation in the 1.35–2, 1–1.35, and 0.5–1 M sucrose interphases, respectively, were diluted to 25 mL with 1.35 M sucrose and centrifuged at 100000*g* for 60 min. The pellets were resuspended in 15 mL of the last solution, and the sus-

pension was subjected to a second gradient centrifugation similar to the first one. The same assays used previously to ascertain the purity of the fractions (Parodi et al., 1984b) (activity of the ovalbumin-dependent galactosyltransferase and RNA/protein ratios) were employed.

Rat liver glucosidase II was prepared as described by Ugalde et al. (1980) up to the lectin affinity step. The enzyme assay described by the same authors was used (Ugalde et al., 1979).

Solubilization and Assays of Glycosyltransferases (Experiment Depicted in Figure 3A). For solubilization of rat liver membrane proteins, the microsomes were treated with 0.6% Triton X-100 and centrifuged at 100000*g* for 60 min. One-third of total protein appeared in the supernatant. Glycosyltransferases were assayed in the untreated membranes and in the detergent-solubilized extract preincubated for different periods at 37 °C as follows:

(*a*) *Urea-Denatured Thyroglobulin Glucosylating Activity.* The incubation mixtures contained, in a total volume of 100 μL, 4 mM Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, 5 μM UDP-[^{14}C]Glc, 50 μM deoxynojirimycin, 480 μg of urea-denatured thyroglobulin, and 100 μg of membrane-bound or soluble rat liver microsomal proteins. Reactions were stopped after 3 min at 37 °C with 1 mL of 10% trichloroacetic acid (see below). Activity obtained with the untreated membranes (100%) corresponds to 2400 cpm. Recovery of total activity in the nonpreincubated soluble extract was 50%.

(*b*) *Dolichol-P-Glc Synthetase.* The incubation mixtures contained, in a total volume of 100 μL, 4 mM Tris-HCl buffer, pH 8.0, 10 mM $MgCl_2$, 0.6% Triton X-100, 5 μM UDP-[^{14}C]Glc, 10 μg of dolichol-P, and 100 μg of membrane-bound or soluble rat liver microsomal proteins. Reactions were stopped after 3 min at 37 °C by the addition of 150 μL of water, 0.4 mL of methanol, and 0.6 mL of chloroform. The lower phases were washed thrice with 0.3 mL of chloroform/methanol/water (1:16:16) and counted. Activity obtained with the untreated membranes (100%) corresponds to 7600 cpm. Recovery of total activity in the nonpreincubated soluble extract was 8%.

(*c*) *Oligosaccharyltransferase.* The incubation mixtures contained, in a total volume of 50 μL, 50 mM Tris–maleate buffer, pH 7.6, 10 mM $MnCl_2$, 0.6% Triton X-100, 2 mM hexapeptide, 50 μM deoxynojirymicin, 10000 cpm of [*glucose*-^{14}C]$Glc_{1-3}Man_9GlcNAc_2$-P-P-dolichol, and 100 μg of membrane-bound or soluble rat liver microsomal proteins. Reactions were stopped after 3 min at 23 °C by the addition of 0.25 mL of methanol/water (1:1). Precipitates were washed with another 0.25 mL of the same solution, the supernatants pooled, and substances therein subjected to paper electrophoresis in 10% formic acid at 25 V/cm for 120 min. Label in positively charged peaks was taken as glycopeptides. Activity obtained with the untreated membranes (100%) corresponds to 2400 cpm. No activity was detected in the nonpreincubated soluble extract.

Thyroglobulin Treatments. Thyroglobulin was subjected to the following treatments:

(*a*) *Treatment with 8 M Urea.* Twenty milligrams of thyroglobulin was dissolved in 1 mL of 10 or 25 mM Tris-HCl buffer, pH 8.0, and dialyzed against 250 mL of 8 M urea and 10 or 25 mM Tris-HCl buffer, pH 8.0 for 5 h after which time the protein was dialyzed against 2 L of the above-mentioned buffers (without urea) for 16 h with a change of buffer after 4 h. Thyroglobulin thus treated will be referred to as urea-denatured thyroglobulin.

(*b*) *Treatment with Endo-β-N-acetylglucosaminidase H.* Ten milligrams of thyroglobulin was dissolved in 1 mL of 25 mM Tris-HCl buffer, pH 8.0, and 8 M urea and dialyzed for 3 h against 250 mL of the same solution and then extensively against 50 mM triethylamine–acetate buffer, pH 5.5. The solution was then divided into halves of 0.5 mL each. To one of them was added 0.005 unit of endo-β-*N*-acetylglucosaminidase H, and both halves were incubated for 24 h at 37 °C, dialyzed against 250 mL of 0.1 M sodium acetate buffer, pH 3.5, and maintained under these conditions for 6 h at 37 °C to inactivate the enzyme (Tarentino & Maley, 1974). Both halves were then dialyzed first against 250 mL of 8 M urea and 25 mM Tris-HCl buffer, pH 8.0, for 4 h and then extensively against the last buffer alone.

(*c*) *Treatment with α-Mannosidase.* Ten milligrams of thyroglobulin was dissolved in 1 mL of 25 mM Tris-HCl buffer, pH 8.0, and dialyzed for 4 h against 250 mL of 8 M urea, 25 mM Tris-HCl buffer, pH 8.0, and then extensively against 50 mM sodium citrate buffer, pH 4.5, and 0.1 mM zinc acetate. The solution was divided into halves of 0.5 mL each, and 1.2 units of jack bean α-mannosidase (previously dialyzed against the last solution) was added to one of them. Both halves were incubated at 37 °C for 20 min after which time they were dialyzed first against 8 M urea and 25 mM Tris-HCl buffer, pH 8.0, and then extensively against the last buffer alone.

(*d*) *Treatment with Nitrous Acid.* Ten milligrams of thyroglobulin was dissolved in 1 mL of 0.1 M sodium acetate buffer, pH 3.5. The solution was divided into halves, and 0.5 mL of 0.33 M sodium nitrite was added to one of them. The other half received 0.5 mL of water. Both halves were incubated at 28 °C for 5 h after which time they were dialyzed first for 4 h against 250 mL of 8 M urea and 25 mM Tris-HCl buffer, pH 8.0, and then extensively against the last buffer alone.

(*e*) *Treatment with Trypsin.* Four milligrams of urea-denatured thyroglobulin was incubated for 60 min at 37 °C in a mixture containing, in a total volume of 375 μL, 8 mM $CaCl_2$, 40 mM Tris-HCl buffer, pH 8.0, and 150 μg of trypsin. The reaction was stopped by the addition of 300 μg of soybean trypsin inhibitor.

Processing of Incubation Mixtures. Incubation mixtures employed are described above or in the text. Except where specifically described, they were processed by three different procedures:

(*a*) Reactions were stopped by the addition of 1 mL of 10% trichloroacetic acid and the suspensions heated for 3 min at 100 °C. After centrifugation, the pellets were twice washed with 1 mL of 10% trichloroacetic acid and counted.

(*b*) Reactions were stopped by the addition of 3–4 volumes of methanol. After centrifugation, the pellets were digested in 3 mL of 0.1 M Tris-HCl buffer, pH 8.0, 2 mM $CaCl_2$, and 2 mg of *S. griseus* protease for 48 h at 37 °C under a toluene atmosphere. The suspensions were then concentrated to 1 mL and centrifuged and the supernatants applied to a 1.2 × 55 cm Sephadex G-10 column equilibrated with 7% 2-propanol. The same solution was used for elution. Fractions containing the glycopeptides (void volume) were pooled, dried, and subjected to paper electrophoresis in 10% formic acid for 120 min at 25 V/cm. Positively charged substances were eluted, treated with endo-β-*N*-acetylglucosaminidase H (0.01 unit in 70 mM triethylamine–acetate buffer, pH 5.5, in a total volume of 350 μL for 20 h at 37 °C), and run again on paper electrophoresis as above. Neutral substances were eluted and run on paper chromatography in 1-propanol/nitromethane/water (5:2:4).

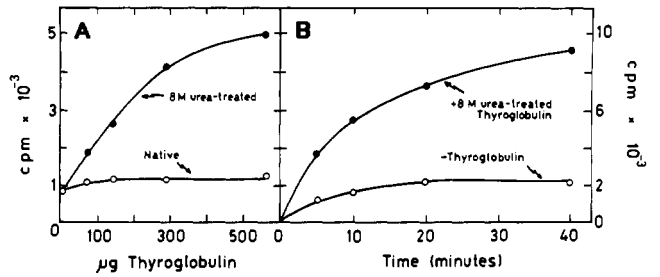

FIGURE 2: Thyroglobulin and time-dependence of protein glucosylation. (A) The incubation mixtures contained, in a total volume of 100 μL, 5 mM Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, 6 μM UDP-[^{14}C]Glc, 200 μg of rat liver microsomal proteins, 200 μM deoxynojirimycin, and the indicated amounts of urea-denatured thyroglobulin (●) or native (○) thyroglobulin (in both cases the protein was dialyzed against 10 mM Tris-HCl buffer, pH 8.0, before the assays). After 4 min at 37 °C, the reactions were stopped with 1 mL of 10% trichloroacetic acid and processed as described under Materials and Methods. (B) The incubation mixtures contained, in a total volume of 100 μL, 10 mM Tris-HCl, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, 3 μM UDP-[^{14}C]Glc, 250 μg of rat liver microsomal proteins, and (●) 150 μg of urea-denatured thyroglobulin. The reactions were stopped after the indicated times at 37 °C with 1 mL of 10% trichloroacetic acid.

(*c*) In the case of incubation mixtures containing trypsinized urea-denatured thyroglobulin, reactions were stopped by heating them at 100 °C for 5 min. Total proteins were degraded with a protease as in procedure b and reaction products quantitiated as glycopeptides after the first electrophoresis.

Methods. Protein was measured by the Lowry method with bovine serum albumin as standard (Lowry et al., 1951). Sugars and glycopeptides were quantitiated by the anthrone–sulfuric acid method with mannose as standard (Dische, 1962).

RESULTS

Stimulation of Protein Glucosylation by Thyroglobulin. Incubation of rat liver microsomes with UDP-[^{14}C]Glc led to the formation of hot 10% trichloroacetic acid insoluble material identified as protein-linked $Glc_1Man_{7-9}GlcNAc_2$ (see below). Addition of native thyroglobulin (dialyzed against 10 or 25 mM Tris-HCl buffer, pH 8.0) to the incubation mixtures in the presence or absence of detergents did not stimulate protein glucosylation (Figure 2A). It is currently accepted that processing of oligosaccharides is stopped at the high-mannose stage when the compounds are inaccessible to processing enzymes (Hieh et al., 1983; Trimble et al., 1983). In the case of thyroglobulin $Man_9GlcNAc_2$ constitutes over 70% of its high-mannose compounds, thus indicating that the latter are particularly hidden from the surrounding milieu (Ito et al., 1977). As this could be the cause of the observed absence of stimulation of protein glucosylation, to open the thyroglobulin molecule and thus render its high-mannose oligosaccharides accessible to glucosyltransferases, the protein was treated with 8 M urea. Thyroglobulin thus treated (urea-denatured thyroglobulin) was found to stimulate up to 10–12-fold protein glucosylation. Curves showing the dependence of protein glucosylation on the addition of native or urea-denatured thyroglobulin and on the incubation time are depicted in Figure 2.

Stimulation by urea-denatured thyroglobulin (but not glucosylation of endogenous rat liver microsomal proteins) required the presence of detergents. Optimal Triton X-100 concentrations ranged between 0.4 and 0.7%. Higher amounts proved to be inhibitory. Other thyroglobulin treatments that were found to stimulate protein glucosylation were S-carboxyamidomethylation, heating at 55 °C, and treatment with 6 M guanidine hydrochloride.

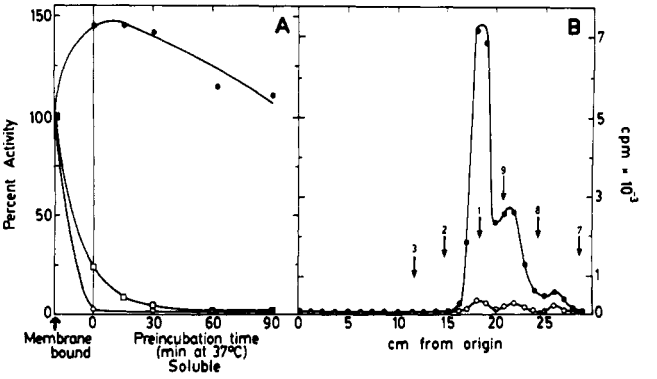

FIGURE 3: Solubilization and stability of glycosyltransferases (A) and identification of reaction products (B). (A) The urea-denatured thyroglobulin glucosylating activity (●), dolichol-P-Glc synthetase (□), and oligosaccharyltransferase (○) were measured by using as sources of enzymes either rat liver microsomal membranes or the detergent-solubilized extract preincubated at 37 °C for the indicated times. Enzyme assays, recovery of protein and enzymatic activities in the soluble extracts, and further experimental details are indicated in a special section under Materials and Methods. (B) The assay of the urea-denatured thyroglobulin glucosylating activity for the soluble enzyme preincubated for 90 min at 37 °C in (A) was scaled up 10-fold in the presence (●) or absence (○) of urea-denatured thyroglobulin. Reactions were stopped by the addition of 3 mL of methanol and processed as described under Materials and Methods: The glycoproteins were treated with a protease, and the glycopeptides thus obtained were treated with endo-β-*N*-acetylglucosaminidase H. Free oligosaccharides were then run on paper chromatography in 1-propanol/nitromethane/water (5:2:4). Standards: 1, $Glc_1Man_9GlcNAc$; 2, $Glc_2Man_9GlcNAc$; 3, $Glc_3Man_9GlcNAc$; 7, $Man_7GlcNAc$; 8, $Man_8GlcNAc$; 9, $Man_9GlcNAc$.

Dolichol Mono- and Diphosphate Derivatives Are Not Involved in Thyroglobulin Glucosylation. Rat liver microsomes were treated with 0.6% Triton X-100 and centrifuged at 100000*g*. About one-third of total protein and half of urea-denatured thyroglobulin glucosylating activity appeared in the supernatant (Figure 3A). The solubilized glucosyltransferase was fairly stable when maintained at 37 °C. These two features (solubilization by detergents and stability at 37 °C) were used to discard the involvement of dolichol mono- and diphosphate derivatives in urea-denatured thyroglobulin glucosylation. Dolichol diphosphate oligosaccharide:polypeptide oligosaccharyltransferase (measured with [*glucose*-^{14}C]-$Glc_3Man_9GlcNAc_2$-P-P-dolichol and Tyr-Asn-Leu-Thr-Ser-Val as substrates) was detected in the untreated membrane fraction but not in the supernatant (Figure 3A). On the other hand, less than 10% of UDP-Glc:dolichol-P glucosyltransferase (measured with UDP-[^{14}C]Glc and dolichol-P as substrates) appeared in the supernatant of the detergent-treated microsomes. This soluble activity was rapidly inactivated at 37 °C, so after 60 min no dolichol-P-Glc formation could be detected (Figure 3A). It may be concluded, therefore, that glucosylation of urea-denatured thyroglobulin did not involve dolichol mono- or diphosphate derivatives as intermediates. Moreover, total inhibition of dolichol-P-Glc formation caused by the addition of amphomycin (400–800 μg/mL) to incubation mixtures containing rat liver membranes as enzyme source was found not to affect urea-denatured thyroglobulin glucosylation (not shown).

Identification of the Reaction Products. That thyroglobulin was actually glucosylated was visualized by autoradiography of a polyacrylamide gel electrophoresis under denaturing conditions of the reaction products. A prominent band migrating as the thyroglobulin monomer (M_r = 330 000) only appeared when urea-denatured thyroglobulin had been added

Table I: Effect of Thyroglobulin Modifications on Protein Glucosylation[a]

expt	additions to incubation mixtures	cpm in 10% trichloroacetic insoluble material
1	none	390
	urea-denatured thyroglobulin treated with endo-β-*N*-acetylglucosaminidase H (212 μg)	410
	urea-denatured thyroglobulin (176 μg)	1080
2	none	200
	urea-denatured thyroglobulin treated with α-mannosidase (128 μg)	210
	urea-denatured thyroglobulin (132 μg)	440
3	none	220
	urea-denatured thyroglobulin treated with nitrous acid (196 μg)	610
	urea-denatured thyroglobulin (208 μg)	600

[a] Above-mentioned treatments of thyroglobulin were performed as described under Materials and Methods. The incubations contained, in a total volume of 100 μL, 0.135 M Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, the indicated amounts of modified or unmodified urea-denatured thyroglobulin, 250 μg of rat liver microsomal proteins, and 3 μM UDP-[^{14}C]Glc. Reactions lasted for 3 min at 37 °C after which time they were stopped by the addition of 1 mL of 10% trichloroacetic acid and processed as described under Materials and Methods.

Table II: Characterization of the Glucose–Mannose Bond in Thyroglobulin-Linked $Glc_1Man_9GlcNAc$ by Inhibition of Rat Liver Glucosidase II[a]

substrate	% inhibition with the 1 mM glucosylglucose inhibitor							
	α(1,2)	α(1,3)	α(1,4)	α(1,6)	β(1,2)	β(1,3)	β(1,4)	β(1,6)
$Glc_1Man_9GlcNAc$ from $Glc_1Man_9GlcNAc_2$-thyroglobulin	16	84	64	6	2	1	2	2
$Glc_1Man_9GlcNAc$ from $Glc_1Man_9GlcNAc_2$-P-P-dolichol	10	79	54	8				
$Glc_2Man_9GlcNAc$ from $Glc_2Man_9GlcNAc_2$-P-P-dolichol	16	84	61	6				

[a] For enzyme source and assay see Materials and Methods.

to the reaction mixture containing membranes as enzyme source (not shown). To further identify the reaction products, detergent-solubilized proteins previously maintained at 37 °C for 90 min were incubated with UDP-[^{14}C]Glc in the presence or absence of urea-denatured thyroglobulin. Total proteins were precipitated with 75% methanol and digested with a protease, and the glycopeptides formed were treated with endo-β-*N*-acetylglucosaminidase H. Oligosaccharides thus liberated were run on paper chromatography. Three peaks appeared, migrating as $Glc_1Man_9GlcNAc$, $Glc_1Man_8GlcNAc$, and $Glc_1Man_7GlcNAc$ standards (Figure 3B) (it has been previously determined that the last two substances migrate between $Man_9GlcNAc$ and $Man_8GlcNAc$ and between $Man_8GlcNAc$ and $Man_7GlcNAc$ standards, respectively). The addition of urea-denatured thyroglobulin to the incubation mixtures drastically increased the amount of the reaction products (Figure 3B). Label appearing in the three above-mentioned oligosaccharides accounted for all radioactivity precipitated by hot trichloroacetic acid. The same results shown in Figure 3B were obtained when rat liver membranes were used as enzyme source. In no case were protein-linked oligosaccharides containing two or three glucose residues detected even though reactions were performed under conditions of total glucosidase I and glucosidase II inhibition (up to 300 μM deoxynojirimycin or the same plus 0.135 M Tris). This precludes the possibility that protein-linked $Glc_1Man_{7-9}GlcNAc_2$ had been produced by successive deglucosylation and demannosylation of a putative protein-linked $Glc_3Man_9GlcNAc_2$.

Further evidence for the involvement of high-mannose oligosaccharides in thyroglobulin as acceptors was obtained when it was found that a previous treatment of urea-denatured thyroglobulin with endo-β-*N*-acetylglucosaminidase H or jack bean α-mannosidase completely abolished the acceptor capacity of the denatured protein (Table I). On the other hand, the stimulation was independent of the presence of Asn residues in urea-denatured thyroglobulin: a previous treatment of the protein with nitrous acid did not modify its effect on protein glucosylation. Besides other modifications of the protein (the latter acquires a deep yellow color) under the conditions employed nitrous acid converts Asn and Gln to Asp and Glu residues, respectively, without affecting high-mannose oligosaccharides (Table I). This confirmed that glucosylation of urea-denatured thyroglobulin was not due to the transfer of an oligosaccharide from a dolichol-P-P derivative to asparagine residues in the protein. The rather low levels of incorporation of label seen in experiments described in Table I were due to the fact that incubation mixtures contained a high concentration of Tris-HCl buffer. These conditions proved to be highly inhibitory not only for glucosidases I and II but also for the urea-denatured thyroglobulin glucosylating activity.

Bond between Glucose and Mannose Residues. We have previously reported that $Glc_1Man_4GlcNAc$ was formed upon endo-β-*N*-acetylglucosaminidase H plus α-mannosidase treatments of protein-linked $Glc_1Man_{7-9}GlcNAc_2$ produced in a variety of intact cells incubated with [^{14}C]glucose or in vitro upon incubation of rat liver or calf thyroid membranes with UDP-[^{14}C]Glc (Parodi & Cazzulo, 1982; Parodi et al., 1983a, 1984b). Moreover, little or no degradation of the $Glc_1Man_4GlcNAc$ thus obtained was observed upon acetolysis of the compound (Parodi et al., 1983b). This indicated that the glucose unit in $Glc_1Man_{7-9}GlcNAc_2$ was linked to the same mannose residue as in dolichol-P-P-linked $Glc_1Man_9GlcNAc_2$. The same results (product of α-mannosidase treatment and its resistance to acetolysis) were obtained with $Glc_1Man_9GlcNAc$ linked to urea-denatured thyroglobulin, but up to now no identification of the bond between the glucose and mannose residues in compounds derived from the glucosylation of glycoproteins has been reported. To elucidate this point, $Glc_1Man_9GlcNAc$ (obtained from urea-denatured thyroglobulin glucosylated under conditions of total glucosidase I and glucosidase II inhibition and in which incorporation into endogenous glycoproteins was at least 10-fold lower) was treated with a partially purified preparation of rat liver glucosidase II in the absence or presence of all possible α- and β-linked glucosyl–glucose disaccharides at 1 mM concentration. As depicted in Table II, glucosyl α(1,3)glucose (nigerose) appeared to be the most potent inhibitor of hydrolysis followed by the α(1,4)-, α(1,2)-, and α(1,6)-linked isomers (maltose, kojibiose, and isomaltose, respectively). None of the β-linked isomers inhibited the reaction. The same inhibition by the α-linked isomers was obtained when dolichol-P-P-linked $Glc_1Man_9GlcNAc_2$ or $Glc_2Man_9GlcNAc_2$ was used as sub-

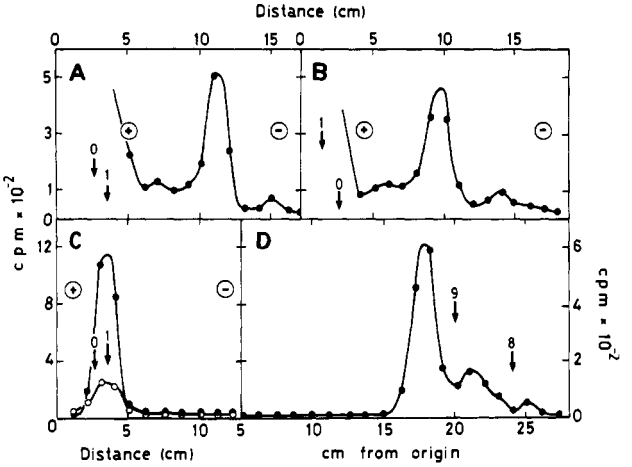

FIGURE 4: Glucosylation of glycopeptides. (A and B) The incubation mixtures contained, in a total volume of 100 μL, 5 mM Tris-HCl buffer, pH 8.0, 5 mM $CaCl_2$, 0.3% Triton X-100, 7.5 μM UDP-[^{14}C]Glc, 300 μM deoxynojirimycin, 250 μg of rat liver microsomal proteins, and glycopeptides equivalent to 18 μg of mannose. In (B) in mixture also contained 40 μg of amphomycin. After 30 min at 37 °C, the reactions were stopped by the addition of 0.4 mL of methanol. The supernatants were saved and the precipitates washed with 0.5 mL of methanol/water (1:1). These supernatants were mixed with the previous ones and dried. The substances therein were subjected to paper electrophoresis in 10% formic acid (120 min at 25 V/cm). (C) The middle (●) and fast (○) migrating substances in (A) and (B) were eluted, pooled, treated with endo-β-*N*-acetylglucosaminidase H, and run on paper electrophoresis as above. (D) The major peak in (C) was eluted and run on paper chromatography in 1-propanol/nitromethane/water (5:2:4). Standards: 9, $Man_9GlcNAc$; 8, $Man_8GlcNAc$; 1, glucose. 0 stands for the origin.

strate [in all cases the reaction products were identified as glucose on paper chromatography in 1-butanol/pyridine/water (6:4:3), and the hydrolysis was inhibited by deoxynojirimycin]. It may be concluded, therefore, that the glucose unit in thyroglobulin-derived $Glc_1Man_9GlcNAc_2$ is linked, the same as in dolichol-bound $Glc_1Man_9GlcNAc_2$ and $Glc_2Man_9GlcNAc_2$, by an α(1,3) bond and that the already characterized glucosidase II is probably responsible for the processing of protein-linked $Glc_1Man_{7-9}GlcNAc_2$. This same approach was used by Ugalde et al. (1980) in the characterization of the glucose bonds in $Glc_{1-3}Man_9GlcNAc_2$-P-P-dolichol.

Glucosylation of Isolated Glycopeptides. High-mannose glycopeptides were obtained by exhaustive degradation of thyroglobulin with an unspecific protease followed by affinity chromatography in concanavalin A–Sepharose. Incubation of the glycopeptide with rat liver membranes and UDP-[^{14}C]Glc in the presence of deoxynojirimycin resulted in the production of labeled substances, soluble in 50% methanol and behaving as cations on paper electrophoresis in 10% formic acid (Figure 4A). No such compounds were formed in the absence of glycopeptides. The same pattern shown in Figure 4A was observed when amphomycin was added to the incubation mixtures (Figure 4B), thus indicating that labeling of the positively charged compounds was independent of dolichol-P-Glc formation (the neutral peaks in Figure 4A,B probably represent labeled glucose produced by decomposition of the substrate). Incubation of the two fast migrating substances with endo-β-*N*-acetylglucosaminidase H resulted in the production of neutral compounds (Figure 4C). Paper chromatography of the major oligosaccharides thus produced revealed the presence of $Glc_1Man_{7-9}GlcNAc$ (Figure 4D).

When label incorporated under identical conditions into the same amounts of oligosaccharides present either in glycopeptides (1–6 μg of mannose) or in urea-denatured thyroglobulin (50–300 μg of protein) was compared, it was found that glucose transfer was at least 2 orders of magnitude faster on the glycoprotein. As no difference in acceptor capacity between urea-denatured thyroglobulin and its tryptic fragments was observed, the results obtained with the unspecific protease-derived glycopeptides suggest that some features in protein domains close to the oligosaccharide-substituted Asn residues are recognized by the glucosyltransferase (results with tryptic fragments are not shown, but conditions of trypsin treatment of urea-denatured thyroglobulin and the glucose transfer assay employed are described under Materials and Methods).

Table III: Subcellular Distribution[a]

membrane	addition of urea-denatured thyroglobulin	cpm in 10% trichloroacetic acid insoluble material
rough endoplasmic reticulum	−	1150
	+	13950
smooth endoplasmic reticulum	−	1650
	+	18550
Golgi apparatus	−	50
	+	2050

[a] The incubation mixtures contained, in a total volume of 100 μL, 10 mM Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, 480 μM deoxynojirimycin, 100–120 μg of the indicated rat liver membrane proteins, 13 μM UDP-[^{14}C]Glc, and, where indicated, 700 μg of urea-denatured thyroglobulin. After 5 min at 37 °C, the incubations were stopped with 1 mL of 10% trichloroacetic acid and processed as indicated under Materials and Methods.

Subcellular Distribution. Incubation of membranes derived from the rat liver rough and smooth endoplasmic reticula and of the Golgi apparatus, UDP-[^{14}C]Glc, and urea-denatured thyroglobulin showed that the endoplasmic reticulum contained most of the glucosylating activity (Table III). It should be noted that the addition of urea-denatured thyroglobulin to the incubation mixtures containing the purified membranes caused a 10–12-fold stimulation of protein glucosylation.

Glucosylation of Thyroglobulin by Plant and Fungal Membranes. Incubation of mung bean membranes with UDP-[^{14}C]Glc did not lead to the glucosylation of endogenous proteins because no positively charged substances appeared upon treatment of all proteins in the incubation mixtures with a protease followed by paper electrophoresis in 10% formic acid (Figure 5A). This was not due to that action of glucosidase II as the assays were performed in the presence of 300 μM deoxynojirimycin. Labeled glycopeptides appeared, however, when urea-denatured thyroglobulin was added to the incubation mixtures (Figure 5B). The neutral substances present in Figure 5A,B were not characterized but probably correspond to a glucan formed by plant enzymes. Upon treatment of the positively charged substances in Figure 5B with endo-β-*N*-acetylglucosaminidase H, all the labeled material was converted to neutral compounds as judged from a second paper electrophoresis in 10% formic acid. The oligosaccharides thus obtained migrated as $Glc_1Man_{7-9}GlcNAc$ on paper chromatography (Figure 5C).

Incubation of *M. rouxii* membranes with UDP-[^{14}C]Glc led to the formation of protein-linked $Glc_1Man_{7-9}GlcNAc_2$. As seen in Figure 5D, addition of urea-denatured thyroglobulin to the incubation mixtures sharply increased the formation of the same substances. It may also be observed that whereas in the absence of exogenous acceptors $Glc_1Man_8GlcNAc_2$ was the major compound formed, in the presence of urea-denatured thyroglobulin the pattern differed and the main product appeared to be $Glc_1Man_9GlcNAc_2$.

Labeling of Thyroglobulin Oligosaccharides by Trypanosomatid Membranes. It has been previously reported that the

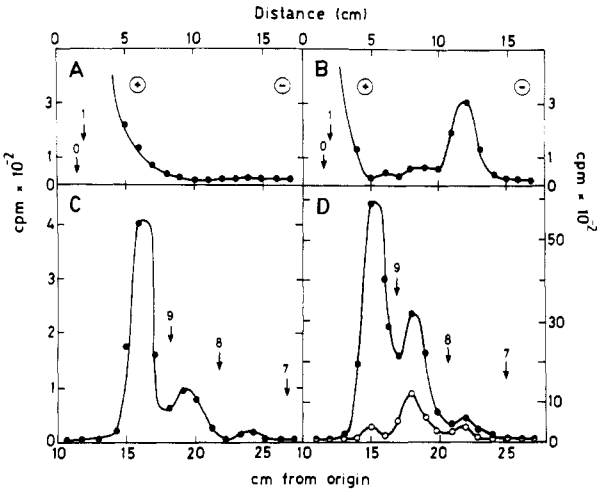

FIGURE 5: Glucosylation of thyroglobulin by plant and fungal membranes. (A and B) The incubation mixtures contained, in a total volume of 1 mL, 10 mM Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, 10 μM UDP-[^{14}C]Glc, 300 μM deoxynojirimycin, and 0.8 mg of mung bean microsomal proteins. In (B) the mixture also contained 3 mg of urea-denatured thyroglobulin. The reactions were stopped after 5 min at 30 °C by the addition of 4 mL of methanol. The precipitates were treated with a protease and the digested substances run on paper electrophoresis in 10% formic acid (120 min at 25 V/cm). (C) The positively charged substances in (B) were treated with endo-β-*N*-acetylglucosaminidase H and run on paper chromatography in 1-propanol/nitromethane/water (5:2:4). (D) Paper chromatography of a sample synthesized and processed as above but with *M. rouxii* membranes in the presence (●) or absence (○) of urea-denatured thyroglobulin. Standards: 9, $Man_9GlcNAc$; 8, $Man_8GlcNAc$; 7, $Man_7GlcNAc$; 1, glucose. 0 stands for the origin.

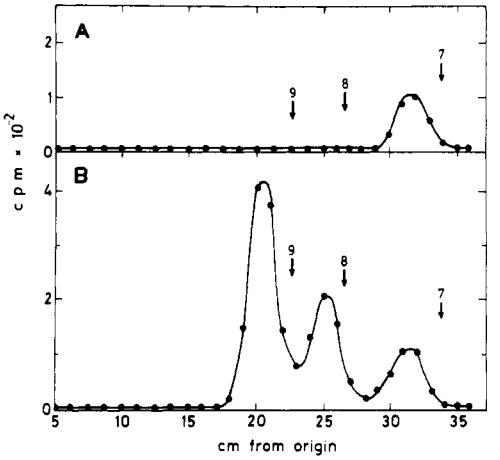

FIGURE 6: Glucosylation of thyroglobulin by *C. fasciculata* membranes. The incubation mixtures contained, in a total volume of 2 mL, 12.5 mM Tris-HCl buffer, pH 8.0, 10 mM $CaCl_2$, 0.6% Triton X-100, 3 μM UDP-[^{14}C]Glc, 100 μM deoxynojirimycin, and 11 mg of *C. fasciculata* membrane proteins. In (B), 6 mg of urea-denatured thyroglobulin was also added. The reactions were stopped after 20 min at 30 °C by the addition of 6 mL of methanol and processed as described under Materials and Methods. The oligosaccharides obtained were run on paper chromatography in 1-propanol/nitromethane/water (5:2:4). Standards: 9, $Man_9GlcNAc$; 8, $Man_8GlcNAc$; 7, $Man_7GlcNAc$.

trypanosomatid protozoan *C. fasciculata*, the same as other microorganisms of the same family, is unable to synthesize dolichol-P-Glc as judged from in vivo and in vitro assays (de la Canal & Parodi, 1987; Mendelzon & Parodi, 1986). Additionally, this species is deficient in the addition of the eighth and ninth mannose residues in the assembly of dolichol-P-P–oligosaccharides, so $Man_7GlcNAc_2$ is transferred in vivo in protein N-glycosylation (de la Canal & Parodi, 1987; Mendelzon & Parodi, 1986; Parodi et al., 1981). Incubation of *C. fasciculata* membranes with UDP-[^{14}C]Glc led to the synthesis of protein-linked $Glc_1Man_7GlcNAc_2$ (Figure 6A), a compound whose formation was previously observed in cells of this parasite incubated with [^{14}C]glucose (Mendelzon & Parodi, 1986). The additional formation of protein-linked $Glc_1Man_9GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ was observed when urea-denatured thyroglobulin was incubated in the cell-free incubation (Figure 6B). Results obtained with microsomal membranes of *T. cruzi* (a parasite transferring $Man_9GlcNAc_2$ in vivo) were similar to those reported for rat liver and *M. rouxii* membranes (Figures 3B and 5D, respectively): addition of urea-denatured thyroglobulin sharply increased the formation of protein-linked $Glc_1Man_{7-9}GlcNAc_2$ (not shown). These results provide further evidence for the glucosylation of high-mannose oligosaccharides in thyroglobulin by a mechanism not involving dolichol-P-Glc.

Discussion

Results presented here show that high-mannose oligosaccharides in denatured thyroglobulin were glucosylated directly from UDP-[^{14}C]Glc when incubated with rat liver membrane, forming protein-linked $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, and $Glc_1Man_7GlcNAc_2$. The first compound appeared to have the same structure as $Glc_1Man_9GlcNAc_2$-P-P-dolichol, thus suggesting that the already characterized glucosidase II is responsible for the processing of the glucosylated glycoproteins formed in vivo (the glucose-mannose bonds in the other two compounds were not characterized, but they are probably the same as in $Glc_1Man_9GlcNAc_2$). The glucose-transferring enzyme appeared to recognize not only the oligosaccharide but also some features of the protein moiety as glycopeptides derived from the digestion of thyroglobulin with an unspecific protease (but not the tryptic fragments) were glucosylated at a much lower rate than denatured thyroglobulin.

Glucosylation of thyroglobulin was maximal in the presence of membranes derived from the rat liver rough and smooth endoplasmic reticula and minimal with material derived from the Golgi apparatus. The crude microsomes used in the present study probably represent the so-called "right side" vesicles as all steps in their preparation were performed in the presence of 0.25 M sucrose. The fact that Triton X-100 at concentrations of 0.4–0.7% was required for thyroglobulin glucosylation suggests that the glucosylating activity resides in the lumen of the endoplasmic reticulum, that is, the same location of protein N-glycosylation and of the initial steps of glycoprotein processing as those catalyzed by glucosidases I and II. The existence in mammalian cells of a system involved in the specific transport of UDP-Glc from the cytoplasm, where it is synthesized, to the lumen of the endoplasmic reticulum, where presumably glucosylation of glycoproteins takes place, has been reported previously (Perez & Hirschberg, 1986). No such transport system was found in Golgi-derived membranes.

The in vivo formation of thyroglobulin-linked $Glc_1Man_{7-9}GlcNAc_2$ in the endoplasmic reticulum of thyroid cells incubated with [^{14}C]glucose has been reported previously, but it was assumed that those compounds had been formed by successive deglucosylation and demannosylation of $Glc_3Man_9GlcNAc_2$ (Godelaine et al., 1981). The fact that thyroglobulin could be glucosylated in vitro only after being treated with 8 M urea suggests that glucosylation of glycoproteins occurs in vivo before glycoproteins adopt their proper folding, a process that precedes their transport to the Golgi apparatus.

Glucosylation of denatured thyroglobulin was also observed when mung bean and *M. rouxii* membranes were used as

enzyme source. We have previously detected the formation of protein-linked $Glc_1Man_{7-9}GlcNAc_2$ in *Phaseolus vulgaris* seeds and *M. rouxii* cells incubated with [^{14}C]glucose, but no clear evidence whether they had been formed by glucosylation of protein-linked $Man_{7-9}GlcNAc_2$ or by successive deglucosylation and demannosylation of protein-linked $Glc_3Man_9GlcNAc_2$ had been obtained (Lederkremer et al., 1986; Parodi et al., 1984b). The present paper shows that glucosylation of glycoproteins also occurs in those systems. Finally, direct transfer of glucose residues from UDP-Glc to high-mannose oligosaccharides in thyroglobulin was demonstrated by using membranes of the trypanosomatid *C. fasciculata*. This protozoan is unable to synthesize dolichol-P-Glc and transfer in vivo $Man_7GlcNAc_2$ in protein N-glycosylation (de la Canal & Parodi, 1987; Mendelzon & Parodi, 1986; Parodi et al., 1981) because it is also deficient in the addition of the eighth and ninth mannose residues in the assembly of dolichol-P-P–oligosaccharides. $Man_7GlcNAc_2$ is therefore the largest high-mannose compound found in *C. fasciculata* glycoproteins (Mendelzon & Parodi, 1986). Incubation of membranes of this parasite with UDP-[^{14}C]Glc led to the production of only protein-linked $Glc_1Man_7GlcNAc_2$, but $Glc_1Man_9GlcNAc_2$ and $Glc_1Man_8GlcNAc_2$ were additionally produced when thyroglobulin was included in the incubation mixture. The glycoprotein glucosylating activity was also detected in microsomal membranes of another trypanosomatid, *T. cruzi*, the causative agent of *Chagas*'s disease. These are the first cell-free demonstrations of the glucosylation of glycoproteins in protozoa.

The widespread distribution of transient glucosylation of glycoproteins suggests an important role for this series of reactions. The rates of transport of different secretory and membrane glycoproteins from the rough endoplasmic reticulum to their final destination in the external milieu or in the plasma membrane may vary by almost an order of magnitude, but the differences among the various glycoproteins reside mainly in the transport from the endoplasmic reticulum to the Golgi cisternae and not in the transit through the latter membranes (Lodish, 1988). On the other hand, it has been reported that addition of deoxynojirimycin (an inhibitor of glycoprotein-processing glucosidases I and II) to various cell systems greatly delayed secretion of most (but not all) glycoproteins (Gross et al., 1983; Lemansky et al., 1984; Lodish & Kong, 1984). Affected glycoproteins were found to accumulate in the endoplasmic reticulum. It was concluded, therefore, that removal of glucose units from protein-linked oligosaccharides was required for an efficient transport of some glycoproteins from the endoplasmic reticulum to the Golgi apparatus. It was later found that deoxynojirimycin also inhibits partially the in vivo formation of dolichol-P-P-linked $Glc_3Man_9GlcNAc_2$ (Romero et al., 1985) (the compound transferred in protein N-glycosylation in mammalian cells), so some of the observed effects of the drug on protein transport might be accounted for by an underglycosylation of glycoproteins rather than by the absence of removal of the glucose units. Nevertheless, it is tempting to speculate that glucosylation of glycoproteins somehow regulates the transport of glycoproteins from the endoplasmic reticulum to the Golgi apparatus.

ACKNOWLEDGMENTS

Radioactive UDP-[^{14}C]Glc was prepared by Susana Raffo and Marta Bravo. Their expertise is gratefully acknowledged. M.B. and S.E.T. are fellows of Merrell Dow International and of the National Research Council (Argentina), respectively, and A.J.P. is a Career Investigator of the latter institution.

REFERENCES

Arima, T., Spiro, M. J., & Spiro, R. G. (1972) *J. Biol. Chem. 247*, 1825–1835.

Bischoff, J., & Kornfeld, R. (1983) *J. Biol. Chem. 258*, 7907–7910.

Bosch, M., Trombetta, S., Engstrom, U., & Parodi, A. J. (1988) *J. Biol. Chem. 263*, 17360–17365.

de la Canal, L., & Parodi, A. J. (1987) *J. Biol. Chem. 262*, 11128–11133.

Dische, Z. (1962) *Methods Carbohydr. Chem. 1*, 478–492.

Godelaine, D., Spiro, M. J., & Spiro, R. G. (1981) *J. Biol. Chem. 256*, 10161–10168.

Gross, V., Andus, T., Tran-Thi, T. A., Schwarz, R. T., Decker, E., & Heinrich, P. C. (1983) *J. Biol. Chem. 258*, 12203–12209.

Hieh, P., Rich Rosner, M., & Robbins, P. W. (1983) *J. Biol. Chem. 258*, 2555–2561.

Ito, S., Yamashita, K., Spiro, R. G., & Kobata, A. (1977) *J. Biochem. (Tokyo) 81*, 1621–1631.

Kornfeld, R., & Kornfeld, S. (1985) *Annu. Rev. Biochem. 54*, 631–664.

Lederkremer, G. Z., & Parodi, A. J. (1986) *Biochim. Biophys. Acta 884*, 363–369.

Lemansky, P., Gieselmann, V., Hasilik, A., & von Figura, K. (1984) *J. Biol. Chem. 259*, 10129–10135.

Lodish, H. F. (1988) *J. Biol. Chem. 263*, 2107–2110.

Lodish, H. F., & Kong, N. (1984) *J. Cell Biol. 98*, 1720–1729.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Mendelzon, D. H., & Parodi, A. J. (1986) *J. Biol. Chem. 261*, 2129–2133.

Mendelzon, D. H., Previato, J. O., & Parodi, A. J. (1986) *Mol. Biochem. Parasitol. 18*, 355–367.

Parodi, A. J. (1979) *J. Biol. Chem. 254*, 8343–8352.

Parodi, A. J., & Cazzulo, J. J. (1982) *J. Biol. Chem. 257*, 7641–7645.

Parodi, A. J., & Quesada-Allue, L. A. (1982) *J. Biol. Chem. 257*, 7637–7640.

Parodi, A. J., Behrens, N. H., Leloir, L. F., & Carminatti, H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3268–3272.

Parodi, A. J., Staneloni, R. J., Cantarella, A. I., Leloir, L. F., Behrens, N. H., Carminatti, H., & Levy, J. (1973) *Carbohydr. Res. 26*, 393–400.

Parodi, A. J., Quesada-Allue, L. A., & Cazzulo, J. J. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6201–6205.

Parodi, A. J., Lederkremer, G. Z., & Mendelzon, D. H. (1983a) *J. Biol. Chem. 258*, 5589–5595.

Parodi, A. J., Medelzon, D. H., & Lederkremer, G. Z. (1983b) *J. Biol. Chem. 258*, 8260–8265.

Parodi, A. J., Martin-Barrientos, J., & Engel, J. C. (1984a) *Biochem. Biophys. Res. Commun. 118*, 1–7.

Parodi, A. J., Mendelzon, D. H., Lederkremer, G. Z., & Martin-Barrientos, J. (1984b) *J. Biol. Chem. 259*, 6351–6357.

Perez, M., & Hirschberg, C. B. (1986) *J. Biol. Chem. 261*, 6822–6830.

Previato, J. O., Mendelzon, D. H., & Parodi, A. J. (1986) *Mol. Biochem. Parasitol. 18*, 343–353.

Rizzolo, L. J., & Kornfeld, R. (1988) *J. Biol. Chem. 263*, 9520–9525.

Romero, P. A., Friedlander, P., & Herscovics, A. (1985) *FEBS Lett. 183*, 29–32.

Tarentino, A. L., & Maley, F. (1974) *J. Biol. Chem. 249*, 811–817.

Trimble, R. B., Maley, F., & Chu, F. K. (1983) *J. Biol. Chem. 258*, 2562-2567.
Ugalde, R. A., Staneloni, R. J., & Leloir, L. F. (1979) *Biochem. Biophys. Res. Commun. 91*, 1174-1181.
Ugalde, R. A., Staneloni, R. J., & Leloir, L. F. (1980) *Eur. J. Biochem. 113*, 97-103.
Wright, A., & Robbins, P. W. (1965) *Biochim. Biophys. Acta 104*, 594-596.

Estrogen Induces N-Linked Glycoprotein Expression by Immature Mouse Uterine Epithelial Cells†

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Received November 29, 1988; Revised Manuscript Received March 3, 1989

ABSTRACT: Characterization of complex glycoconjugates and the effects of estrogen on their expression in immature mouse uterine epithelial cells are reported. The secreted fraction contained nonanionic, O-linked lactosaminoglycan (LAG)-bearing proteins of M_r 30 000–40 000 as well as anionic, O-linked, LAG-bearing glycoproteins with very high apparent molecular weight (>670K). Heparan sulfate (HS) proteoglycans and HS linked to little or no protein were found in the secreted fraction as well. A very similar array of glycoconjugates was found in the nonhydrophobic fraction of cell-associated macromolecules. In addition, the hydrophobic cell-associated fraction contained nonanionic, LAG-bearing glycoproteins of approximately 250K, anionic LAG-bearing glycoproteins distributing over a wide range of molecular weights, and HS proteoglycans with median molecular weights of approximately 250K. In contrast to the glycoproteins produced by their mature counterparts, virtually all glycoproteins produced by immature cells were O-linked. Estrogen treatment of immature mice caused uterine epithelial cells to secrete anionic, high molecular weight (>670K) N-linked glycoproteins as a major product. These estrogen-responsive glycoproteins did not appear to contain LAGs. Estrogen treatment also markedly decreased the proportion of all hydrophobic glycoconjugates in the cell-associated fraction. Collectively, these observations indicate that one aspect of the estrogen-induced maturation of uterine epithelial cells is the stimulation of N-linked glycoprotein synthesis and secretion. Furthermore, stimulation of N-linked glycoprotein synthesis by itself is insufficient to support N-linked LAG glycoprotein production.

Alterations in glycoprotein expression have been associated with a variety of stimuli and developmental processes. A particularly interesting example of this is embryo implantation into the uterus. Both morphological (Lee et al., 1983) and biochemical (Farach et al., 1987) studies indicate that marked changes in the pattern of glycoconjugate expression occur at both embryonic and uterine cell surfaces during mouse embryo implantation. Furthermore, it appears that glycoconjugates directly participate in cell recognition/adhesion events that occur in this system (Farach et al., 1987; Dutt et al., 1987; Carson et al., 1987a). It has been well documented that expression of a uterine state that is permissive or receptive to embryo implantation is strictly controlled by steroid hormones (Psychoyos, 1973). Consequently, these hormones also should influence the expression of uterine glycoconjugates involved in embryo implantation. Uterine cells do not retain their steroid responsiveness in vitro (Glasser, 1985). Consequently, model systems for the study of uterine steroid hormone responses have been animals without endogenous sources of steroids, i.e., immature or castrate adult. Injection of these animals with particular steroids defines their hormonal status, and the subsequent responses can be studied in tissue explants or primary cultures. Using this approach in adult mice, it has been shown that estrogens markedly stimulate N-linked glycoprotein synthesis (Dutt et al., 1986, 1988) as well as dolichol-linked oligosaccharide assembly (Carson et al., 1987b) in uteri; however, it is not clear which uterine cell types are involved in this response. It also appears that estrogen has anabolic effects with regard to glycoprotein expression since turnover of heparan sulfate proteoglycans of uterine epithelial cells is stimulated by estrogen (Morris et al., 1988). Thus, estrogens exert their effects on uterine glycoprotein expression at a number of levels.

One interesting class of uterine N-linked glycoproteins are those bearing lactosaminoglycans (LAGs).[1] These polysaccharides have been detected through the use of the LAG-degrading enzyme endo-β-galactosidase (Fukuda, 1985) and the LAG-specific lectin pokeweed mitogen (Irimura & Nicolson, 1983). Uterine LAG synthesis in ovariectomized mature mice is preferentially stimulated by estrogens (Dutt et al., 1988). Furthermore, it has been shown that LAGs are major cell surface polysaccharides of uterine epithelial cells where they participate in aspects of cell adhesion (Dutt et al., 1987). Heparan sulfate proteoglycans (HSPGs) also are expressed at the cell surface of both the uterine epithelium and periimplantation-stage blastocysts (Farach et al., 1987; Tang et al., 1987). The blastocyst HSPGs appear to participate in

† This work was supported by grants from the National Institutes of Health (HD-18768) and the March of Dimes (1-958) awarded to D.D.C.
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[1] Abbreviations: E_2, 17β-estradiol; HS, heparan sulfate; LAG, lactosaminoglycan.

0006-2960/89/0428-8116$01.50/0