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Recognition of the Oligosaccharide and Protein Moieties of Glycoproteins by the UDP-Glc:Glycoprotein Glucosyltransferase†

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Received May 9, 1991; Revised Manuscript Received August 12, 1991

ABSTRACT: It was found, in cell-free assays, that the $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ isomers having the mannose unit to which the glucose is added were glucosylated by the rat liver glucosyltransferase at 50 and 15%, respectively, of the rate of $Man_9GlcNAc_2$ glucosylation. This indicates that processing by endoplasmic reticulum mannosidases decreases the extent of glycoprotein glucosylation. All five different glycoproteins tested (bovine and porcine thyroglobulins, phytohemagglutinin, soybean agglutinin, and bovine pancreas ribonuclease B) were found to be poorly glucosylated or not glucosylated unless they were subjected to treatments that modified their native conformations. The effect of denaturation was not to expose the oligosaccharides but to make protein determinants, required for enzymatic activity, accessible to the glucosyltransferase because (a) cleavage of denatured glycoproteins by unspecific (Pronase) or specific (trypsin) proteases abolished their glucose acceptor capacities almost completely except when the tryptic peptides were held together by disulfide bonds and (b) high mannose oligosaccharides in native glycoproteins, although poorly glucosylated or not glucosylated, were accessible to macromolecular probes as concanavalin A-Sepharose, endo-β-*N*-acetylglucosaminidase H, and jack bean α-mannosidase. In addition, denatured, endo-β-*N*-acetylglucosaminidase H deglycosylated glycoproteins were found to be potent inhibitors of the glucosylation of denatured glycoproteins. It is suggested that in vivo only unfolded, partially folded, and malfolded glycoproteins are glucosylated and that glucosylation stops upon adoption of the correct conformation, a process that hides the protein determinants (possibly hydrophobic amino acids) from the glucosyltransferase.

The N-glycosylation of protein is initiated by the transfer of an oligosaccharide having in most cases the composition $Glc_3Man_7GlcNAc_2$ from a dolichol-P-P derivative to asparagine residues in nascent or recently terminated polypeptide chains. The transfer reaction occurs in the rough endoplasmic reticulum and is immediately followed by removal of the glucose units catalyzed by two specific glucosidases: glucosidase I, which removes the more external unit, and glucosidase II, which excises both remaining units. Several of the peripheral α(1,2)-linked mannose residues may be then removed by two α-mannosidases located, the same as for both glucosidases, in the lumen of the endoplasmic reticulum. Further

† This work was supported by United States Public Health Service Grant 1 RO1 GM44500-01, by the National Research Council (Argentina), by the University of Buenos Aires, and by the Swedish Agency for Research Cooperation with Developing Countries.
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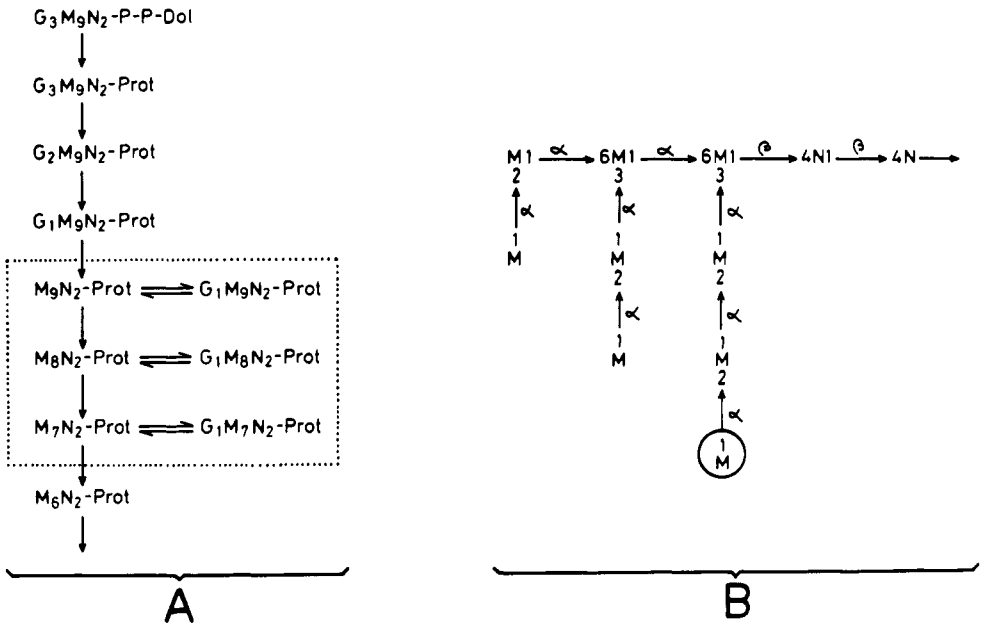

FIGURE 1: (A) Processing of oligosaccharides occurring in the endoplasmic reticulum. Reactions within the square represent the so-called transient glucosylation of glycoproteins. (B) Structure of $Man_9GlcNAc_2$. The mannose unit to which the glucose is transferred directly from UDP–Glc in an $\alpha(1,3)$ linkage is depicted within a circle. G, M, N, Prot, and Dol stand for glucose, mannose, *N*-acetylglucosamine, protein, and dolichol, respectively.

processing of the oligosaccharide occurs in the Golgi apparatus (Kornfeld & Kornfeld, 1985).

We have previously reported that glucose-free, N-linked oligosaccharides may be transiently glucosylated in the endoplasmic reticulum of mammalian, plant, fungal, and protozoan cells (Bosch et al., 1988; Lederkremer & Parodi, 1986; Mendelzon & Parodi, 1986; Parodi et al., 1983a,b, 1984). The reaction products appeared to be, in most cases, protein-linked $Glc_1Man_{7-9}GlcNAc_2$. The structure of the $Glc_1Man_9GlcNAc_2$ thus formed appeared to be identical with that of $Glc_1Man_9GlcNAc_2$–P-P-dolichol. This indicates that glucosidase II is also responsible for the deglucosylation of the newly glucosylated compounds. The enzyme involved in the transfer reaction (UDP–Glc:glycoprotein glucosyltransferase) was detected in microsomal membranes of rat liver, mung bean, *Mucor rouxii*, *Trypanosoma cruzi*, and *Crithidia fasciculata* cells (Trombetta et al., 1989). The assays involved incubation of microsomal membranes, calcium ions, UDP–[^{14}C]Glc, and 8 M urea-denatured thyroglobulin as glucose acceptor. The native form of the glycoprotein was ineffective. The sugar donor of the transfer reaction was UDP–Glc, and no dolichol derivatives were involved in it (Trombetta et al., 1989). Further work showed that the glucosyltransferase was not a membrane-bound protein but a soluble protein of the lumen of the endoplasmic reticulum (Trombetta et al., 1991). Oligosaccharide processing reactions occurring in the endoplasmic reticulum are depicted in Figure 1A. Those within the dotted square represent the so-called transient glucosylation of glycoproteins. The structure of $Man_9GlcNAc_2$ is shown in Figure 1B. The mannose unit to which the glucose is transferred directly from UDP–Glc in an $\alpha(1,3)$ linkage is drawn within a circle. It is worth stressing the fact that the same structure is found in $Glc_1Man_9GlcNAc_2$–P-P-dolichol.

About 50% of all N-linked oligosaccharides were found to be transiently glucosylated in vivo in the protozoa *T. cruzi* and *C. fasciculata* (Gañán et al., 1991; Gotz et al., 1991). As the average number of oligosaccharides per molecule of glycoprotein is higher than one, this indicates that more than 50% of the total glycoproteins (perhaps all of them) are glucosylated and that transient glucosylation is a major event in the normal processing of glycoproteins. It may be assumed that a similar proportion of N-linked oligosaccharides is transiently glucosylated in other eukaryotic cells, but there is no evidence for this assertion. On the other hand, the fact that not all oligosaccharides are glucosylated suggested the existence of some sort of modulation of glycoprotein glucosylation.

As mentioned above, denatured but not native thyroglobulin was glucosylated in cell-free assays. It was suggested that the effect of denaturation was to make the oligosaccharides accessible to the glucosyltransferase (Trombetta et al., 1989). Evidence is presented in this paper showing this suggestion not to be correct. The effect of glycoprotein denaturation appeared to be to expose certain protein domains, the interaction of which with the glucosyltransferase is apparently required for the transfer reaction to occur. In addition, results presented indicate that processing of oligosaccharides by endoplasmic reticulum mannosidases strongly influences the extent of glycoprotein glucosylation.

Experimental Procedures

Materials. Porcine and bovine thyroglobulins, thyroglobulin–Sepharose, bovine serum albumin, bovine pancreas ribonuclease B (type III-B), endo-β-*N*-acetylglucosaminidase H (endo H),[1] *Streptomyces griseus* protease (type XIV or Pronase), bovine pancreas trypsin (type XIII), soybean trypsin inhibitor (type I-S), 1-deoxynojirimycin, castanospermine, and jack bean α-mannosidase were from Sigma (St. Louis, MO). Concanavalin A–Sepharose was from Pharmacia (Uppsala, Sweden). Tritiated sodium borohydride (8 Ci/mmol) was from Amersham (U.K.). UDP–[^{14}C]Glc (285 Ci/mol) was prepared according to Wright and Robbins (1965) with slight modifications.

Preparation of Lectins. Soybean agglutinin was prepared as described by Gordon et al. (1972). The lectin was further purified by affinity chromatography in acid-treated Sepharose-4B (Allen & Johnson, 1976; Jaffe et al., 1977). Phytohemagglutinin was essentially prepared as described by Felsted

[1] Abbreviation: endo H, endo-β-*N*-acetylglucosaminidase H.

et al. (1975). The final purification step was an affinity chromatography in thyroglobulin–Sepharose. Both lectins appeared to be homogeneous as judged by polyacrylamide gel electrophoresis under denaturing conditions.

UDP–Glc:Glycoprotein Glucosyltransferase. Three different preparations were employed: (a) rat liver microsomal membranes were prepared as described previously (Parodi et al., 1972). Their protein content was 30–50 mg/mL. This preparation was used in experiments described in Figure 2. (b) The soluble rat liver glucosyltransferase membrane preparation was subjected to high pressure with a French press and centrifuged at 100000*g* for 60 min as described by Trombetta et al. (1991). Ammonium sulfate (up to 50%) was added to the supernatant, and proteins thus precipitated were dialyzed against 10 mM Tris-HCl buffer, pH 7.6/5 mM 2-mercaptoethanol. The final protein concentrations were 5–10 mg/mL. This preparation was used in experiments described in Figure 4 and Tables I–IV, except for experiment 3 in Table II. (c) The rat liver soluble preparation (20–50 mL) was applied to a 10 × 1.5 cm DEAE–cellulose column equilibrated with 10 mM Tris-HCl buffer, pH 7.6/5 mM 2-mercaptoethanol, and the enzyme was eluted with 150 mL of a 0–0.5 M NaCl gradient in the same solution. The enzyme was eluted at about 0.3 M NaCl and was dialyzed as above. The final protein concentration was 1–5 mg/mL. This was the only preparation that was devoid of endogenous glycoprotein acceptors and was used in experiment 3 in Table II.

Standards. [*glucose*-^{14}C]$Glc_1Man_{7-9}GlcNAc$ was prepared by incubating 8 M urea-denatured thyroglobulin with UDP-[^{14}C]Glc and rat liver microsomal membranes, followed by a protease and endo H treatments of proteins as described before (Trombetta et al., 1989). [^{14}C]$Man_{7-9}GlcNAc$ was prepared from hen oviduct slices incubated with [^{14}C]glucose as described previously (Parodi et al., 1981). Reduction of the oligosaccharides with $NaBH_4$ produced the standards $Man_9GlcNAcol$, $Man_8GlcNAcol$, and $Man_7GlcNAcol$. Acetolysis of them yielded, among other products, $Man_4GlcNAcol$ and $Man_3GlcNAcol$.

Glucosyltransferase Assays. The incubation mixtures employed are described elsewhere in the text. Three different procedures were employed for quantitating label in reaction products: (a) for the hot 10% trichloroacetic acid method, reactions were stopped by the addition of 1 mL of 10% trichloroacetic acid. Tubes were heated for 2 min at 100 °C and the precipitates were washed three times with the same acid and counted. This method was employed when incorporation into glycoproteins was to be measured. (b) For measuring the incorporation into tryptic peptides (all experiments in Table II), reactions were stopped by the addition of 1 mL of water. Tubes were then heated for 5 min at 100 °C. Proteins and peptides were then degraded with *S. griseus* protease (0.2 mg) in 1.2 mL of 0.15 M Tris-HCl buffer, pH 8.0/2 mM $CaCl_2$. Incubations lasted for 16 h at 37 °C. The solutions were then applied to a Sephadex G-10 column (1.2 × 57 cm) equilibrated with 7% 2-propanol. Glycopeptides appeared in the void volume. They were submitted to paper electrophoresis in 10% formic acid (25 V/cm for 120 min). Labeled glycopeptides migrated about 8–11 cm to the cathode. (c) For measuring incorporation into glycopeptides obtained by degradation of the thyroglobulins with *S. griseus* protease, reactions were stopped by the addition of 1 volume of methanol. Supernatants were withdrawn, and the precipitates were washed twice with 0.5 mL of methanol/water (1:1). All three supernatants were mixed and submitted to paper electrophoresis as above.

Preparation of Pronase-Derived Glycopeptides. Glycopeptides from bovine and porcine thyroglobulins were obtained as described before, but after a first incubation with *S. griseus* protease the tubes were heated for 5 min at 100 °C and centrifuged at low speed. The supernatants were applied to a 1.2 × 57 cm Sephadex G-10 column equilibrated with 7% 2-propanol. The glycopeptides appeared in the void volume and were subjected to a second protease digestion. The purification procedure was continued as already described (Trombetta et al., 1989).

Trypsin Degradation. The incubation mixtures contained, in a total volume of 0.4 mL, 3.5 mg of the corresponding denatured glycoprotein, 10 mM $CaCl_2$, 0.2 mg of trypsin, and 10 mM Tris-HCl buffer, pH 8.0. Soybean trypsin inhibitor (0.52 mg) was added after 60 min at 37 °C. In the controls the inhibitor was added before the protease.

Reduction of Thyroglobulin. The reduction was performed in 8 M urea/1 mM NaEDTA, pH 7.7/20 mM dithiothreitol for 16 h at 37 °C. The glycoprotein was then dialyzed against 10 mM Tris-HCl buffer, pH 8.0/5 mM 2-mercaptoethanol. In the case of denatured, trypsin-treated thyroglobulin, the urea–EDTA–dithiothreitol-containing solution was applied to a 1.2 × 57 cm Sephadex G-10 column equilibrated with 7% 2-propanol/5 mM 2-mercaptoethanol. Glycopeptides appeared in the void volume. They were dried and dissolved in 10 mM Tris-HCl buffer, pH 8.0/5 mM 2-mercaptoethanol.

Glycoprotein Denaturation Procedures. Denaturation of thyroglobulins was performed as already described; that is, the glycoproteins were solubilized in 10 mM Tris-HCl buffer, pH 8.0, first dialyzed against 8 M urea for 4 h and finally against the buffer solution. In the case of phytohemagglutinin, soybean agglutinin, and bovine pancreas ribonuclease B, the glycoproteins in the urea solution were heated for 4 h at 60 °C. In experiments described in Table IV and Figure 4, all glycoproteins were dialyzed against 50 mM triethylamine acetate buffer, pH 7.0 before the glucosyltransferase assays.

Methods. Acetolysis was performed as described before (Engel & Parodi, 1985). Protein was measured as described by Lowry et al. (1951) with bovine serum albumin as the standard. Thyroglobulin-derived glycopeptides were quantitated as described by Dische (1962) with mannose as the standard. Chromatographies and electrophoresis were performed on Whatman 1 papers. The solvents used were (A) 1-propanol/nitromethane/water (5:2:4), (B) 1-butanol/pyridine/water (4:3:4), and (C) 1-butanol/ethanol/water (4:1:1).

Influence of the Primary Sequence of the Oligosaccharides on the Glucosylation Rate (Figure 2). The glucosylating incubation mixtures contained, in a total volume of 100 μL, 10 mM $CaCl_2$, 5 mM Tris-HCl buffer, pH 8.0, 300 μM castanospermine, 6 μM UDP-[^{14}C]Glc, 0.6% Triton X-100, 12 μg of porcine or bovine thyroglobulin-derived glycopeptides, and 0.8 mg of rat liver microsomal proteins. The incubation mixtures were processed as described above (method c) after 15 and 30 min at 37 °C. Incorporation of label into glycopeptides was proportional to incubation periods. Positively charged glycopeptides were treated with endo H (5 milliunits in 200 μL of 50 mM triethylamine acetate buffer, pH 5.5, for 16 h at 37 °C) and run on paper chromatography. The oligosaccharide substrates were tritium-labeled as follows: 20 μg of glycopeptides was treated with endo H as above, dried, and dissolved in 1.5 mL of 10 mM sodium borate buffer, pH 11.3. Solid labeled sodium borohydride was added to the solution. After 16 h at room temperature, the solution was acidified with 1 M acetic acid and dried. Methanol (1 mL) and a drop of glacial acetic acid were added, and the solution

was dried. This step was repeated two more times. The substances were then dissolved in water and passed through a 0.5 × 3 cm Dowex 50 (proton form) column, and the solution was dried. The methanol/acetic acid step was then performed two more times. Substances in the sample were then subjected to paper chromatography with solvent C. Substances in the origin were then eluted and run on paper chromatography with solvent A.

RESULTS

Influence of the Primary Sequence of Oligosaccharides on the Glucosylation Rate. In order to study the influence of the primary sequence of oligosaccharides on the glucosylation rate without any possible interference from the protein moiety, the following experiments were conducted using, as glucose acceptors, the glycopeptides produced by exhaustive degradation of porcine and bovine thyroglobulins with an unspecific protease. Yamashita et al. (1978) determined that a single asparagine residue remained linked to the high mannose oligosaccharide when ovalbumin was degraded under the conditions employed here. In order to ensure that a single amino acid (or, at most, a limited number of them) remained linked to the oligosaccharides, the degradation procedure described by Yamashita et al. (1978) was performed twice, first on thyroglobulin and then on the resulting glycopeptides (see Experimental Procedures). Glycopeptides thus obtained were incubated with UDP-[^{14}C]Glc and rat liver microsomal membranes, and the incubations were stopped with 50% methanol. Substances in the supernatants were run on paper electrophoresis in 10% formic acid. It is worth mentioning that incubation mixtures contained 300 μM castanospermine and that rat liver glucosidase II is completely inhibited at this concentration of the drug.

Positively charged substances in the electrophoresis were eluted, treated with endo H, and run on paper chromatography (parts A and B of Figure 2 are patterns obtained from glucosylated porcine and bovine glycopeptides, respectively). In order to visualize the pattern of the acceptor substrates employed, the unlabeled glycopeptides obtained by exhaustive degradation of both thyroglobulins were treated with endo H, reduced with tritiated sodium borohydride, and run on paper chromatography (parts C and D of Figure 2 are patterns obtained from porcine and bovine glycopeptides, respectively). Comparison of panels A and C with B and D showed striking differences. For instance, in both substrate samples $Man_7GlcNAc$ constituted a relatively high proportion of the oligosaccharides (Figure 2C,D), but very small amounts of $Glc_1Man_7GlcNAc$ were obtained with both glycopeptide acceptors (Figure 2A,B). This could be due to the fact that processing had removed from $Man_7GlcNAc$ (and $Man_8GlcNAc$) the mannose units to which the glucose residues are added in the glucosylation reaction. Refer to Figure 1B to better understand this and further points raised below.

Quantitation of the proportion of the $Man_8GlcNAcol$ and $Man_7GlcNAcol$ isomers having the suitable acceptor structure was performed by subjecting them (and $Man_9GlcNAcol$) to acetolysis. This is a chemical reaction that breaks preferentially $\alpha(1,6)$ bonds between mannose units. As the oligosaccharides were labeled at the reducing ends, it should have been expected that the only labeled material yielded by $Man_9GlcNAcol$ was $Man_4GlcNAcol$, whereas $Man_8GlcNAcol$ and $Man_7GlcNAcol$ could have produced $Man_4GlcNAcol$ and $Man_3GlcNAcol$, the former fragment being yielded by the isomers able to accept glucose units. The pattern obtained upon acetolysis of $Man_9GlcNAcol$ (from porcine thyroglobulin) is depicted in Figure 2E. Although the main product

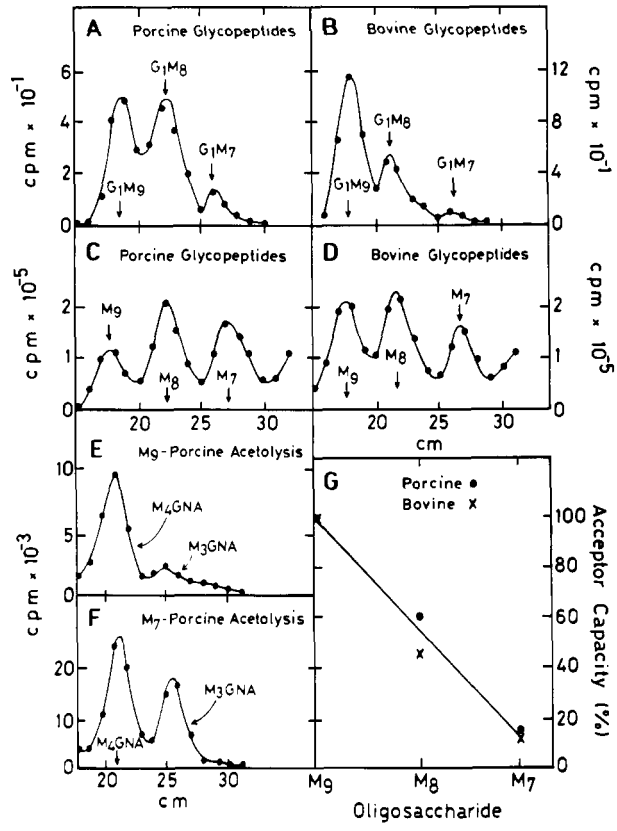

FIGURE 2: The influence of the primary sequence of oligosaccharides on the glucosylation rate. Panels A and B: Glycopeptides obtained by exhaustive degradation of porcine (A) and bovine (B) thyroglobulins with an unspecific protease were incubated with rat liver glucosyltransferase and UDP-[^{14}C]Glc for 30 min. Glucosylated glycopeptides were treated with endo H and run on paper chromatography (solvent A). Panels C and D: The porcine (C) and bovine (D) glycopeptide substrates employed in panels A and B were treated with endo H, reduced with tritiated sodium borohydride, and run on paper chromatography (solvent A). Panels E and F: substances migrating as the $Man_9GlcNAcol$ (E) and $Man_7GlcNAcol$ (F) standards in C were subjected to acetolysis and then to paper chromatography (solvent B). Panel G: The acceptor capacities of the asparagine-linked $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ isomers having the mannose residue to which the glucose unit is added were calculated as indicated in the text. The acceptor capacity of $Man_9GlcNAc_2$-Asn derived from porcine or bovine thyroglobulins was taken as 100%. Standards: G_1M_9, $Glc_1Man_9GlcNAc$; G_1M_8, $Glc_1Man_8GlcNAc$; G_1M_7, $Glc_1Man_7GlcNAc$; M_9, $Man_9GlcNAcol$; M_8, $Man_8GlcNAcol$; M_7, $Man_7GlcNAcol$; M_4GNA, $Man_4GlcNAcol$; M_3GNA, $Man_3GlcNAcol$. For further details see Experimental Procedures.

was, as expected, $Man_4GlcNAcol$, smaller fragments produced by unspecific breakdown appeared in the chromatogram (this is a common result of acetolysis reactions). Nevertheless, acetolysis of $Man_8GlcNAcol$ and $Man_7GlcNAcol$ produced much higher proportions of $Man_3GlcNAcol$ than $Man_9GlcNAcol$. For instance, the pattern obtained upon acetolysis of $Man_7GlcNAcol$ (prepared from porcine thyroglobulin) is shown in Figure 2F. Data obtained from acetolysis of $Man_9GlcNAcol$, $Man_8GlcNAcol$, and $Man_7GlcNAcol$ from both thyroglobulins allowed calculation of the proportion of the $Man_8GlcNAcol$ and $Man_7GlcNAcol$ isomers in parts C and D of Figure 2 having the correct acceptor structures. The percentage of each of the reaction products (Figure 2A,B) as well as that of each of the oligosaccharide substrates having the proper acceptor structures (Figure 2C,D, corrected by acetolysis data) allowed calculation of the relative acceptor capacities of $Man_8GlcNAc_2$ (50%) and $Man_7GlcNAc_2$ (15%) ($Man_9GlcNAc_2$ = 100) (Figure 2G). This result indicates that processing of oligosaccharides by the endoplasmic reticulum

Table I: Effect of Glycoprotein Denaturation on the Glucose Acceptor Capacity

addition to incubation mixtures	cpm in glycoprotein[a]	wavelength[b] (nm)
none	110	
phytohemagglutinin, native	535	332
phytohemagglutinin, denatured	2348	339
soybean agglutinin, native	195	328
soybean agglutinin, denatured	6275	340
bovine thyroglobulin, native	421	332
bovine thyroglobulin, denatured	4082	341
porcine thyroglobulin, native	262	332
porcine thyroglobulin, denatured	2969	340
none	366	
ribonuclease B, native	477	278.2; 286.5
ribonuclease B, denatured	1748	277.6; 284.6

[a] The glucose acceptor capacities of glycoproteins were assayed in incubation mixtures that contained, in a total volume of 100 μL, 10 mM $CaCl_2$, soluble rat liver glucosyltransferase (30–50 μg of protein), 20 mM Tris–HCl buffer, pH 8.0, 2 μM UDP–[^{14}C]Glc, 200 μM castanospermine, and 250–400 μg of the indicated glycoproteins. After 5 min at 37 °C, the label in material insoluble in hot 10% trichloroacetic acid was quantitated. [b] In the case of the first four glycoproteins, the values represent the wavelengths of maximal fluorescent emission (excitation wavelength 280 nm) (Teale, 1960), and in the case of ribonuclease B, the values represent the wavelengths of two peaks of the fourth derivative of the tyrosine ultraviolet absorption spectrum (Padrós et al., 1982).

α-mannosidases strongly affects the glucosylation of glycoproteins even if the mannose units to which the glucose residues are added are conserved.

Effect of Denaturation on the Acceptor Capacity of Glycoproteins. Most of the following experiments were performed with soluble preparations of the UDP–Glc:glycoprotein glucosyltransferase. As mentioned above, the enzyme appeared to be a soluble protein of the lumen of the endoplasmic reticulum (Trombetta et al., 1991). Practically all the activity can be solubilized upon disruption of a rat liver microsomal fraction with a French press, in the absence of detergents (see Experimental Procedures). As all dolichol-dependent glycosyltransferases remain membrane-bound after the high-pressure treatment, this clearly rules out the involvement of lipid derivatives in the observed glucose transfer reactions. On the other hand, the exclusive occurrence of a direct transfer from the sugar nucleotide to glycoproteins was demonstrated when rat liver microsomes were incubated under the conditions employed here (Trombetta et al., 1989).

In addition to bovine thyroglobulin, which was employed before (Trombetta et al., 1989), four other glycoproteins (porcine thyroglobulin, phytohemagglutinin, soybean agglutinin, and bovine pancreas ribonuclease B) were tested as acceptors of glucose units. In all cases the native forms of the glycoproteins were very poorly glucosylated or not glucosylated at all (depending on the assays) by the rat liver glucosyltransferase, whereas their denatured forms always served efficiently as glucose acceptors (Tables I, III, and IV). The changes in protein conformation that elicited the glucose acceptor capacities were monitored by the shift in the wavelength of maximal fluorescent emission of tryptophan in the case of phytohemagglutinin, soybean agglutinin, and both thyroglobulins (Teale, 1960). In the case of ribonuclease B, a protein not having that residue, the conformational change was followed by the shift in the wavelength of two peaks of the fourth derivative of the ultraviolet absorption spectrum. Those peaks correspond to the maximum and the shoulder values of tyrosine absorption (Padrós et al., 1982). The shifts in wavelength values shown in Table I indicate that tryptophan and tyrosine are in a more hydrophilic environment after the denaturation treatments. It is worth mentioning that the treatment that produced the change in conformation and elicited the glucose acceptor capacity in both thyroglobulins (dialysis against 8 M urea for 4 h at room temperature, see Experimental Procedures) did not modify either of these properties in phytohemagglutinin, soybean agglutinin, and ribonuclease B. In the case of these glycoproteins it was necessary to heat the urea-containing solution for 4 h at 60 °C. In all cases, a change in protein conformation was required for the appearance of the glucose acceptor capacity.

Table II: Effect of Trypsin Digestion on Glycoprotein Glucosylation[a]

experiment	glycoprotein added	cpm in reaction product
1	none	843
	phytohemagglutinin (denatured)	4039
	phytohemagglutinin (denatured + trypsin)	363
2	none	854
	soybean agglutinin (denatured)	6244
	soybean agglutinin (denatured + trypsin)	358
3	none	0
	thyroglobulin (denatured)	1594
	thyroglobulin (denatured + trypsin)	1536
	thyroglobulin (denatured and reduced)	1425
	thyroglobulin (denatured and reduced + trypsin)	0
4	none	626
	thyroglobulin (denatured)	3593
	thyroglobulin (denatured + trypsin)	3752
	thyroglobulin (denatured + trypsin, then reduced)	162

[a] The glucose acceptor capacities of glycoproteins were assayed in incubation mixtures containing, in a total volume of 100 μL, 10 mM $CaCl_2$, 1 mM castanospermine, 0.5 mg of the glycoprotein indicated, 6 μM UDP–[^{14}C]Glc, 4 mM Tris-HCl buffer, pH 8.0, and soluble rat liver glucosyltransferase (30–50 μg of protein) (experiments 1, 2, and 4) or the same but purified by DEAE–cellulose chromatography (5 μg of protein) (experiment 3). After 5 min at 37 °C, samples were processed according to method b. For further details see Experimental Procedures.

Table III: Accessibility of Oligosaccharides in Native Glycoproteins to Concanavalin A–Sepharose

experiment	addition to incubation mixtures	cpm in reaction products[a]	percent glycoprotein bound to lectin[b]
1	none	375	
	phytohemagglutinin (native)	482	100
	phytohemagglutinin (denatured)	4012	100
2	none	201	
	soybean agglutinin (native)	205	100
	soybean agglutinin (denatured)	5631	100
3	none	912	
	thyroglobulin (native)	921	100
	thyroglobulin (denatured)	5842	100

[a] The glucose acceptor capacities of glycoproteins were assayed in incubation mixtures containing, in a total volume of 100 μL, 5 mM $CaCl_2$, 7 mM Tris-HCl buffer, pH 8.0, 370 μM castanospermine, 8 μM UDP–[^{14}C]Glc, 0.5 mg of the glycoprotein indicated, and soluble rat liver glucosyltransferase (30–50 μg of protein). After 5 min at 37 °C, the label in substances insoluble in hot 10% trichloroacetic acid was quantitated. [b] Parallel tubes only containing the glycoproteins were diluted with 0.5 mL of 0.15 M NaCl/50 mM Tris-HCl buffer, pH 7.6/1 mM $CaCl_2$/1 mM $MgCl_2$/1 mM $MnCl_2$. The same volume of packed concanavalin A–Sepharose suspended in the same solution was added to the tubes. After 10 min at 37 °C, protein was assayed in the supernatants of a low-speed centrifugation.

Table IV: Accessibility of Oligosaccharides in Native Glycoproteins to Endo H and α-Mannosidase[a]

experiment	addition to incubation mixtures	cpm in reaction products
1	none	2976
	phytohemagglutinin (native)	3544
	phytohemagglutinin (native + endo H, then denatured)	5013
	phytohemagglutinin (denatured)	16182
	phytohemagglutinin (denatured + endo H)	759
2	none	1237
	thyroglobulin (native)	1189
	thyroglobulin (native + endo H, then denatured)	1275
	thyroglobulin (denatured)	4865
	thyroglobulin (denatured + endo H)	628
3	none	1428
	thyroglobulin (native)	1403
	thyroglobulin (native + α-mannosidase, then denatured)	2270
	thyroglobulin (denatured)	6219
	thyroglobulin (denatured + α-mannosidase)	1957

[a] The glucose acceptor capacities of glycoproteins were assayed in incubation mixtures containing, in a total volume of 100 μL, 8 mM $CaCl_2$, 1.3 mM castanospermine, soluble rat liver glucosyltransferase (30–50 μg of protein), 20 mM triethylamine acetate buffer, pH 7.0, 4 μM UDP[^{14}C]Glc, and 0.8 mg of the glycoproteins indicated. After 5 min at 37 °C, the label in substances insoluble in hot 10% trichloroacetic acid was quantitated. The deglycosylated species were prepared by first incubating native or denatured glycoproteins (6–10 mg) in 0.5 mL of 50 mM triethylamine acetate buffer, pH 7.0, with 20 milliunits of endo H (experiments 1 and 2) or in 50 mM sodium acetate buffer, pH 5.9/0.5 mM zinc acetate with 1.6 units of jack bean α-mannosidase (experiment 3) for 6 h at 37 °C followed by a denaturation cycle. For further details see Experimental Procedures.

Assays shown in Table I were quantitated by the hot 10% trichloroacetic acid method (see Experimental Procedures). As described before (Trombetta et al., 1989), all labeled material insoluble in this acid was protein-linked $Glc_1Man_9GlcNAc_2$, $Glc_1Man_8GlcNAc_2$, and $Glc_1Man_7GlcNAc_2$ when the incubations were performed in the absence of denatured glycoproteins. Addition of both thyroglobulins or of ribonuclease B to the incubation mixtures stimulated synthesis of the same products, whereas soybean agglutinin or phytohemagglutinin only stimulated formation of the first or of the first and second compounds, respectively. Procedures followed for the identification of the reaction products are fully described in Trombetta et al. (1989).

Rate of Glucosylation of Pronase-Derived Glycopeptides. It was mentioned previously that high mannose oligosaccharides present in glycopeptides obtained by exhaustive degradation of bovine thyroglobulin with an unspecific protease (Pronase) were glucosylated at a rate that was more than 100-fold slower than that of the same compounds present in the denatured glycoprotein, but no experimental results were presented (Trombetta et al., 1989). The initial rates of glucosylation of increasing amounts of glycopeptides or of thyroglobulin are now depicted in Figure 3. It was found that about 2% of the total weight in bovine thyroglobulin may be accounted for by high mannose type oligosaccharides (Trombetta et al., 1989). This value agrees with that reported by Arima et al. (1972).

Assays using both acceptor substrates were somewhat different. In the case of denatured thyroglobulin label in hot 10% trichloroacetic acid, insoluble material was measured. As this included glycoproteins present in the enzyme preparation that were glucosylated in the assay, incorporation in the absence of thyroglobulin was not null. On the other hand, incorporation into glycopeptides was measured as labeled material soluble in 50% methanol and running to the cathode in electrophoresis in 10% formic acid. In this case, no incorporation was detected in the absence of added glycopeptides.

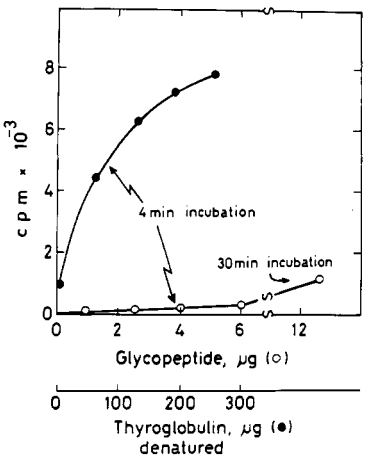

FIGURE 3: Initial rates of glucosylation of oligosaccharides linked to thyroglobulin or to asparagine residues. The indicated amounts of denatured bovine thyroglobulin or of glycopeptides obtained by degradation of the same glycoprotein with *S. griseus* protease were incubated for 4 min (or 30 min where indicated) at 37 °C with 0.7% Triton X-100, 10 mM $CaCl_2$, 100 μM 1-deoxynojirimycin, 4 mM Tris-HCl buffer, pH 8.0 0.6 mg of rat liver microsomal membrane proteins, and 3 μM UDP-[^{14}C]Glc in a total volume of 100 μL. The label in the glycoprotein or in the glycopeptides was quantitated by methods a and c, respectively (see Experimental Procedures).

Glucosylation of Trypsin-Derived Glycopeptides. It was mentioned previously that a trypsin treatment of denatured thyroglobulin did not modify its glucose acceptor capacity (Trombetta et al., 1989) (no experimental results were provided). It was therefore suggested that the interaction of a protein determinant, close in the amino acid sequence to the oligosaccharide, with the glucosyltransferase was required for the occurrence of the transfer reaction (Trombetta et al., 1989). Work reported below shows this interpretation not to be correct.

Denatured phytohemagglutinin, soybean agglutinin, and bovine thyroglobulin were treated with chymotrypsin-free trypsin, and the digests thus obtained were tested as glucose acceptors. Assays in these cases were different from those mentioned above: incubations (100 μL) were stopped by the addition of 1 mL of water and the tubes were heated for 5 min at 100 °C. Proteins and peptides were then degraded with an unspecific protease, and the glycopeptides thus produced were desalted by gel filtration and subjected to paper electrophoresis in 10% formic acid. Positively charged substances were quantitated.

Phytohemagglutinin and soybean agglutinin gave similar results: the tryptic glycopeptides were not only not glucosylated but glucosylation of endogenous glycoproteins present in the enzyme preparations was somewhat inhibited by the tryptic digest (Table II, experiments 1 and 2). Results of experiment 1 in Table II were exactly the same whether both the trypsin digestions and the glucosyltransferase assays were performed in the absence or presence of 0.15% Triton X-100.

A different result was obtained with bovine thyroglobulin: as reported before (Trombetta et al., 1989), the tryptic digest of denatured thyroglobulin incorporated the same amount of glucose as the undegraded, denatured glycoprotein (experiment 3 in Table II). Contrary to phytohemagglutinin and soybean agglutinin, which have no disulfide bridges, thyroglobulin is formed by two monomers of 330 kDa, linked by several of the above mentioned bonds. Each monomer has 5–6 high mannose

type oligosaccharides. The denatured monomer (denatured and reduced thyroglobulin, experiment 3 in Table II) served as acceptor the same as the denatured dimer, but the tryptic digest of the monomer was not glucosylated. This is consistent with results obtained with both lectins.

Two possible explanations may be envisaged for the absence of an effect of trypsin digestion on the incorporation into the denatured dimer: (a) trypsin did not cleave the glycoprotein or, alternatively, (b) the protease did effectively cleave the glycoprotein but the disulfide bridges kept a certain tertiary structure that allowed glucosylation. Experiment 4 in Table II showed the latter possibility to be true: the trypsin-treated dimer could be glucosylated, but it lost its acceptor capacity if subsequently reduced.

Results in Table II reinforce those obtained before with Pronase-derived glycopeptides as they show that high mannose oligosaccharides that were completely exposed to the glucosyltransferase as those in tryptic glycopeptides were not glucosylated.

Both phytohemagglutinin and soybean agglutinin have a single high mannose type oligosaccharide per molecule. The tryptic fragment of the last lectin, which was not glucosylated, contains, according to the known amino acid sequence of the glycoprotein, 12 and 9 amino acids linked to the amino and carboxyl groups of the carbohydrate-bearing asparagine, respectively (Adar et al., 1989). In the tryptic glycopeptides of phytohemagglutinin, the corresponding values are 1 and 8. This rules out the possibility that the poor glucosylation of Pronase-derived glycopeptides could be due to a requirement of amino acid substitutions in the asparagine unit for the glucosylation reaction to occur. As mentioned above, it was previously suggested that the glucosyltransferase somehow recognized a structural feature in the protein backbone vicinal to the oligosaccharide (Trombetta et al., 1989). Results shown in Table II make highly unlikely this suggestion, and they rather indicate that the protein domains interacting with the transferase could be distantly located, in the amino acid sequence, from the oligosaccharide: the tryptic peptides of proteins devoid of disulfide bonds were not glucosylated, but the glucose transfer reaction occurred if the oligosaccharides and the putative interacting domains were kept close by the above-mentioned linkages. It is worth mentioning that in experiment 3 in Table II a glucosyltransferase preparation not having endogenous glycoprotein acceptors was employed (see Experimental Procedures).

Exposure of High Mannose Type Oligosaccharides in Native Glycoproteins. Experiments in Table III show that although the denatured but not the native forms of bovine thyroglobulin, phytohemagglutinin, and soybean agglutinin were glucosylated by rat liver glucosyltransferase, both forms of the glycoproteins were recognized by convanavalin A–Sepharose (in the case of thyroglobulin binding was abolished if 0.1 M methyl α-mannoside was included in the binding assay).

Another macromolecular probe used for testing exposure of high mannose type oligosaccharides in native glycoproteins was endo H. Native phytohemagglutinin and bovine thyroglobulin were incubated with endo H, subjected then to denaturation conditions, and tested as glucose acceptors (it was verified that endo H was inactivated by the denaturing treatment).

As shown in Table IV, glucosylation of phytohemagglutinin and thyroglobulin was strongly affected when the endo H treatments were performed on native glycoproteins, thus indicating that their oligosaccharides were at least partially

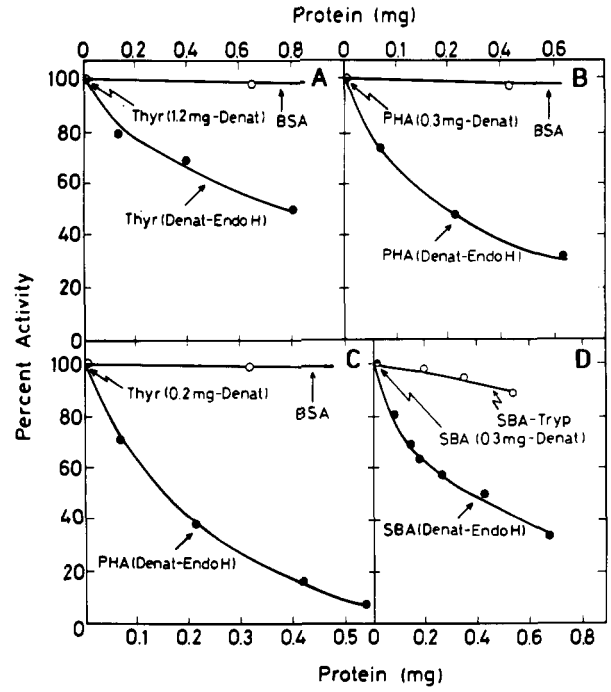

FIGURE 4: Inhibition of glucosylation of denatured glycoproteins by denatured, deglycosylated species. The indicated amounts of denatured bovine thyroglobulin (A, Thyr), phytohemagglutinin (B, PHA), and soybean agglutinin (D, SBA) were incubated with soluble rat liver glucosyltransferase (30–50 μg of protein), 8 μM UDP-[^{14}C]Glc, 5 mM $CaCl_2$, 400 μM castanospermine, 35 mM triethylamine acetate buffer, pH 7.0, and increasing amounts of the same species first denatured and then endo H-deglycosylated or bovine serum albumin (BSA), in a total volume of 100 μL. After 5 min at 37 °C the label in material insoluble in hot 10% trichloroacetic was counted. In panel C the incubation mixtures contained denatured bovine thyroglobulin (0.2 mg) and increasing amounts of denatured, deglycosylated phytohemagglutinin. In panel D, instead of BSA, increasing amounts of a tryptic digest of denatured soybean agglutinin (SBA–Tryp) were added. The deglycosylated species were prepared as described in the legend to Table IV. For further details see Experimental Procedures.

accessible to the glycosidase. The partial accessibility of high mannose oligosaccharides in native thyroglobulin to endo H has been previously reported by Trimble and Maley (1984). It is worth mentioning that no proteolytic activity was detected in the endo H preparation as judged by polyacrylamide gel electrophoresis of endo H treated phytohemagglutinin under denaturing conditions. On the other hand, Tarentino et al. (1974) have shown that the single oligosaccharide present in bovine pancreas ribonuclease B is fully accessible to endo H in the native form of the enzyme and, as shown in Table I, that species is a very poor glucose acceptor.

Treatment of native bovine thyroglobulin with jack bean α-mannosidase strongly diminished the acceptor capacity of the glycoprotein when it was denatured after the enzymatic degradation (Table IV, experiment 3). This result reinforces those mentioned above as it indicates that oligosaccharides in native thyroglobulin that were not glucosylated by the glucosyltransferase were nevertheless accessible to a macromolecular probe.

Effect of Deglycosylated Glycoproteins and of Saccharides on the Glucosylation Reaction. As shown in Figure 4, glycoproteins first denatured and then endo H deglycosylated inhibited glucosylation of the denatured glycosylated species. Thus, deglycosylated thyroglobulin inhibited glucosylation of the same glycosylated glycoprotein (Figure 4A), deglycosylated phytohemagglutinin inhibited that of glycosylated phytohemagglutinin, and thyroglobulin (parts B and C of Figure 4, respectively), and deglycosylated soybean agglutinin inhibited

that of the same denatured glycosylated lectin (Figure 4D). This was not due to an unspecific protein effect because, as shown above, the native forms of glycoproteins did not affect glucosylation of endogenous glycoprotein acceptors present in the enzyme preparation and, in addition, bovine serum albumin did not affect glucose incorporation into denatured glycosylated glycoproteins (Figure 4A–C). Moreover, the tryptic peptides obtained upon digestion of denatured soybean agglutinin only slightly inhibited glucosylation of the denatured glycosylated glycoprotein (Figure 4 D). The tryptic peptides were separated on HPLC, and pools of 3–4 of them were tested as possible inhibitors of the lectin glucosylation, but as was the same as with the whole mixture of them, none of the peptide pools had a significant effect. On the other hand, and in sharp contrast with the inhibition produced by the denatured deglycosylated protein backbones, no inhibition of the glucosylation of denatured soybean agglutinin was observed with a 60- or 120-fold molar excess of methyl α-mannoside or a 4-fold molar excess of high mannose, asparagine-linked oligosaccharides.

Discussion

As mentioned above, previous results showed that probably only about half of all N-linked oligosaccharides are transiently glucosylated in vivo in the endoplasmic reticulum. This strongly suggests the existence of some sort of modulation mechanism of the glucosylation reaction. Results presented here show that processing of glycoproteins by endoplasmic reticulum α-mannosidases strongly affects the glucosylation rate. $Man_8GlcNAc_2$ and $Man_7GlcNAc_2$ isomers having the mannose unit to which the glucose is attached were glucosylated at 50 and 15%, respectively, of the $Man_9GlcNAc_2$ glucosylation rate. The fact that such minor modifications in the primary structure of $Man_9GlcNAc_2$ produce sharp decreases in its glucose acceptor capacity may reflect the dramatic changes in conformation triggered by removal of the terminal nonreducing mannose units other than that to which the glucose residue is added (Bischoff et al., 1986; Wooten et al., 1990). The Manα(1,6)Manβ linkage shows a much higher flexibility in the main $Man_8GlcNAc_2$ isomer produced by endoplasmic reticulum α-mannosidases than in $Man_9GlcNAc_2$. Consequently, both oligosaccharides may adopt very different conformations. Similarly, removal of the other terminal mannose unit increases the flexibility of the Manα(1,6)Manα bond whereas removal of the mannose residue to which the glucose unit is added does not significantly affect the conformation of the oligosaccharide. A similar influence of the primary sequence of the oligosaccharide on the activity of rat liver glucosidase II has been reported: it was found that $Glc_1Man_8GlcNAc$ and Glc_1Man_7NAc were deglucosylated, in cell-free assays, at 21 and 9%, respectively, of the rate of $Glc_1Man_9GlcNAc$ deglucosylation (Grinna & Robbins, 1980).

Concerning the protein backbone, results presented here suggest the presence in them of determinants whose interaction with the glucosyltransferase is required for the occurrence of an efficient transfer reaction. Those determinants appeared to be exposed in denatured but not in native glycoproteins. The effect of denaturation was not to make the oligosaccharides accessible to the glucosyltransferase because (a) Pronase- or trypsin-derived glycopeptides in which the oligosaccharides are supposed to be freely accessible to the transferring enzyme were very poorly glucosylated and (b) oligosaccharides in native glycoproteins were accessible to such macromolecular probes as concanavalin A–Sepharose, endo H, and α-mannosidase. In addition to those two facts, evidence for the presence of determinants in the protein backbone was afforded by the effect of trypsin on denatured thyroglobulin, a glycoprotein formed by two monomers linked by disulfide bonds: the denatured monomers and dimers and the trypsin-treated dimers were glucosylated whereas the trypsin-treated monomers and the dimers first treated with trypsin and later reduced were not. This suggests that the spatial proximity of the putative determinants and the oligosaccharides is required for the glucosyl transfer reaction. Separation of the determinants and the oligosaccharides by proteolytic cleavages abolished glucosylation unless both elements were held close by disulfide bridges. Tryptic digests of glycoproteins somewhat inhibited glucosylation of endogenous glycoproteins present in the enzyme preparations. This might be due to the presence of inhibitory protein domains among the generated peptides.

Further evidence for the presence, in denatured glycoprotein backbones, of determinants interacting with the glucosyltransferase was provided by the fact that denatured, endo H deglycosylated glycoproteins were potent inhibitors of the glucosylation of denatured glycoproteins. More than 50% inhibition was attained in the presence of equimolecular amounts of glycosylated and deglycosylated species. Binding of the protein determinants in deglycosylated glycoproteins to the glucosyltransferase would be expected to inhibit glucosylation of glycosylated species. As mentioned above, the effect of deglycosylated species was specific as it was not observed when native glycoproteins or bovine serum albumin were added to the incubation mixtures. Whereas denatured deglycosylated species strongly inhibited glucosylation of glycoproteins, no effect of methyl α-mannoside and asparagine-linked high mannose oligosaccharides was detected. The concentrations of those compounds in the assays were 60–120- and 4-fold higher, respectively, than that of the high mannose oligosaccharide linked to the acceptor glycoprotein.

The characteristics of the interaction of the glucosyltransferase with denatured glycoproteins highly resemble that of the UDP–GlcNAc:lysosomal enzyme *N*-acetylglucosaminylphosphotransferase with lysosomal enzymes (Lang et al., 1984). This transferase very poorly phosphorylates glycopeptides and oligosaccharides and is highly dependent on the conformation of the lysosomal enzymes for activity. Modifications of the native three-dimensional structures of the glycoprotein substrates by limited proteolysis or by heat results in a loss of affinity. Moreover, endo H deglycosylated lysosomal enzymes inhibit phosphorylation of the glycosylated ones.

Several hypotheses may be advanced concerning the nature of the protein determinants interacting with the glucosyltransferase. They may be formed by (a) specific amino acid sequences common to all glycoproteins; (b) certain amino acids that are separated in the primary sequence but that become close in the denatured conformation; and (c) not specific amino acids but common three-dimensional structures shared by all denatured glycoproteins but generated by totally different amino acid sequences. The widespread distribution in nature of transient glucosylation indicates that very different glycoproteins are glucosylated in vivo. Moreover, as described here, totally unrelated denatured glycoproteins, such as both lectins, the thyroglobulins, and ribonuclease B, or related glycoproteins but having different conformations as denatured dimeric and monomeric thyroglobulin, all apparently share the same determinants. The variety of glycoproteins that are glucosylated makes implausible, therefore, all the hypotheses advanced above as it should not be reasonably expected for any of the structural features alluded in them to be common to all glucosylated glycoproteins. Therefore, the nature of the protein determinants remains unclear.

The interiors of water-soluble proteins in their native states are predominantly composed of hydrophobic amino acids while the hydrophilic side chains are on the exterior where they interact with water. Denatured states have, in general, more hydrophobic side chains on the exterior than native ones. As mentioned above, the wavelength shifts shown in Table I indicate that the tryptophan and tyrosine residues are in a more hydrophilic environment after the denaturing treatments. It could be, therefore, that the effect of denaturation is to provide a certain hydrophobic environment in the vicinity of the oligosaccharide and that this environment is required for the activity of the glucosyltransferase. The fact that the tryptic peptides obtained from denatured soybean agglutinin did not significantly affect glucosylation of the denatured lectin suggests that the individual peptides are not able to provide the hydrophobic environment present in the surface of denatured proteins.

Suh et al. (1989) showed that the G glycoprotein of a thermosensitive vesicular stomatitis virus mutant remained in the endoplasmic reticulum at the nonpermissive temperature because it could not trimerize due to its aberrant conformation. Trimerization is required for the passage of the G glycoprotein to the Golgi apparatus. The presence, at the nonpermissive temperature, of a glucose unit in the high mannose oligosaccharide of the G glycoprotein was detected. It was determined that this monosaccharide unit had been added posttranslationally, most probably by a reaction catalyzed by the UDP–Glc:glycoprotein glucosyltransferase. Moreover, a turnover of the glucose unit was demonstrated, thus confirming a shuttle between the monoglucosylated and unglucosylated states of the glycoprotein. According to the high molar percentage of N-glycoproteins that are glucosylated in vivo under normal conditions (Gañán et al., 1991; Gotz et al., 1991) it cannot be expected for transient glucosylation to be restricted to malfolded structures. It may be assumed that unfolded or partially folded ones are also glucosylated and that glucosylation only stops once glycoproteins adopt their proper tertiary structures, that is, conformations in which the putative protein determinants are hidden from the glucosyltransferase.

The enzyme appears to be, therefore, a sensor of untrimmed oligosaccharides and of unfolded/malfolded structures and might be somehow related to the mechanism by which glycoproteins fold correctly or to that by which cells recognize and subsequently degrade malfolded structures in the endoplasmic reticulum.

ACKNOWLEDGMENTS

The expertise of Ms. Susana Raffo in the preparation of labeled UDP–Glc is gratefully recognized.

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