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A Major Proportion of N-Glycoproteins Are Transiently Glucosylated in the Endoplasmic Reticulum†

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ABSTRACT: N-Linked, high-mannose-type oligosaccharides lacking glucose residues may be transiently glucosylated directly from UDP-Glc in the endoplasmic reticulum of mammalian, plant, fungal, and protozoan cells. The products formed have been identified as N-linked $Glc_1Man_{5-9}GlcNAc_2$ and glucosidase II is apparently the enzyme responsible for the in vivo deglucosylation of the compounds. As newly glucosylated glycoproteins are immediately deglucosylated, it is unknown whether transient glucosylation involves all or nearly all N-linked glycoproteins or if, on the contrary, it only affects a minor proportion of them. In order to evaluate the molar proportion of N-linked oligosaccharides that are glucosylated, cells of the trypanosomatid protozoan *Trypanosoma cruzi* (a parasite transferring $Man_9GlcNAc_2$ in protein N-glycosylation) were grown in the presence of [^{14}C]glucose and concentrations of the glucosidase II inhibitors deoxynojirimycin and castanospermine that were more than 1000-fold higher than those required to produce a 50% inhibition of the *T. cruzi* enzyme. About 52-53% of total N-linked oligosaccharides appeared to have glucose residues. The compounds were identified as $Glc_1Man_{7-9}GlcNAc_2$. The same percentage was obtained when cells were pulsed-chased with [^{14}C]glucose in the presence of deoxynojirimycin for 60 min. No evidence for the presence of an endomannosidase yielding GlcMan from the glucosylated compounds was obtained. As the average number of N-linked oligosaccharides per molecule in glycoproteins is higher than one, these results indicate that more than 52-53% of total glycoproteins are glucosylated and that transient glucosylation is a major event in the normal processing of glycoproteins.

N-Glycosylation is initiated in most eukaryotes by the transfer of $Glc_3Man_9GlcNAc_2$ from a dolichol-P-P derivative to asparagine residues in polypeptide chains (Kornfeld & Kornfeld, 1985). This reaction, which occurs in the lumen of the rough endoplasmic reticulum, is immediately followed by the removal of the three glucose units from the protein-linked oligosaccharides. Two specific glucosidases are involved in the initial steps of oligosaccharide processing: glucosidase I, which cleaves the more external $\alpha(1,2)$-linked unit, and glucosidase II, which removes the remaining $\alpha(1,3)$-linked glucose residues. Two specific $\alpha(1,2)$-mannosidases located, as are both glucosidases, in the lumen of the endoplasmic

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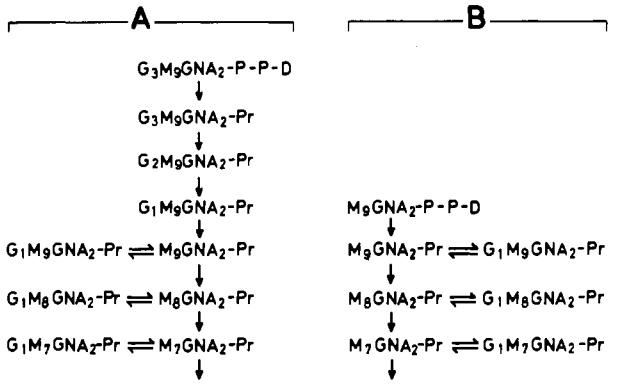

FIGURE 1: Processing of *N*-linked oligosaccharides. The processing occurring in the endoplasmic reticulum of mammalian (A) or *T. cruzi* cells (B) is shown. D stands for dolichol, P_r for protein, M for mannose, G for glucose, and GNA for N-acetylglucosamine.

reticulum, may then remove several of the peripheral mannose units from the oligosaccharides (Bischoff & Kornfeld, 1983; Bischoff et al., 1986; Rizzolo & Kornfeld, 1988). Further processing of the oligosaccharides takes place in the Golgi apparatus.

Trypanosomatid protozoa are unique in nature as they are the only wild-type eukaryotes known to transfer unglucosylated oligosaccharides in protein N-glycosylation ($Man_6GlcNAc_2$, $Man_7GlcNAc_2$, and $Man_9GlcNAc_2$, depending on the species) (Bosch et al., 1988b). A reaction first detected in those parasites and later in mammalian, plant, and fungal cells incubated with [^{14}C]glucose is the transient glucosylation of protein-linked, glucose-free, high-mannose-type oligosaccharides (Lederkremer & Parodi, 1986; Mendelzon & Parodi, 1986; Parodi et al., 1983a,b, 1984b). The reaction products appeared in most cells to be protein-linked $Glc_1Man_{7-9}GlcNAc_2$ but $Glc_1Man_{5,6}GlcNAc_2$ were detected in protozoa transferring $Man_6GlcNAc_2$ to protein (Mendelzon et al., 1986; Parodi et al., 1984a). In all cases the glucosylated compounds disappeared upon chasing the cells with unlabeled glucose.

A cell-free assay for the glycoprotein glucosylating activity was developed (Trombetta et al., 1989). UDP-Glc was found to be the glucosyl donor. No dolichol derivatives were involved in the transfer reaction and the enzyme was found to be located in the rough and smooth endoplasmic reticula and to require calcium ions for activity. Denatured thyroglobulin was used as an acceptor in the assay as the native form was ineffective. The enzyme (UDP-Glc:glycoprotein glucosyltransferase) was thus detected in rat liver, mung bean, *Mucor rouxii*, *Trypanosoma cruzi*, and *Crithidia fasciculata* microsomal membranes (Trombetta et al., 1989). Furthermore, the structure of thyroglobulin-linked $Glc_1Man_9GlcNAc_2$ was found to be identical with that of $Glc_1Man_9GlcNAc_2$-P-P-dolichol, thus suggesting that the already characterized glucosidase II is also responsible for the in vivo deglucosylation of the newly glucosylated compounds. The processing of the N-linked oligosaccharides occurring in the endoplasmic reticulum of mammalian and *T. cruzi* cells is depicted in parts A and B of Figure 1, respectively.

In spite of the wide distribution of transient glucosylation of glycoproteins in nature, no role for this reaction has been found yet. Moreover, as glucosylated glycoproteins are immediately deglucosylated in vivo, it is even unknown if direct glucosylation from UDP-Glc affects all or nearly all N-linked glycoproteins or only a minor proportion of them. Suh et al. (1989) have recently shown that the G protein of a thermosensitive vesicular stomatitis virus mutant (ts045) remained within the endoplasmic reticulum at the nonpermissive temperature. The oligosaccharides of this glycoprotein were of the high mannose type and contained a single glucose unit that had been added posttranslationally, most probably by the mechanism of glucosylation described in our laboratory. The oligosaccharides were processed to complex-type compounds upon lowering the temperature, thus indicating that the glycoprotein had been transferred to the Golgi apparatus and that the glucose units had been removed. The oligosaccharides of G proteins of other thermosensitive mutant virus that also remained in the endoplasmic reticulum at the nonpermissive temperature did not contain glucose units. A known difference between the G protein of ts045 and of the other mutant virus was that only the G protein from the former virus could not trimerize due to its aberrant conformation at the nonpermissive temperature. Trimerization is a process that precedes exit of the G protein from the endoplasmic reticulum. The authors suggested that glucosylation of glycoproteins might be part of the mechanism by which cells recognize aberrant/malfolded structures. As it is not expected that cells would normally form a high proportion of malfolded glycoproteins, this suggestion is only compatible with a low proportion of N-glycoproteins being glucosylated.

In order to evaluate the molar proportion of N-linked oligosaccharides that are glucosylated, a similar approach previously used by Bischoff et al. (1986) for assessing the role of an endoplasmic reticulum α-mannosidase in the processing of N-linked oligosaccharides was employed; that is, cells were grown in the presence of substances interferring with the metabolic step whose significance was under study. The structures of the N-linked oligosaccharides thus obtained were then compared with those of compounds synthesized under normal conditions.

Results presented herein indicate that transient glucosylation of glycoproteins is a major event in the normal processing of glycoproteins.

Experimental Procedures

Materials. [^{14}C]Glucose (250–300 Ci/mol) was from American Radiolabeled Chemicals. Deoxynojirimycin (DNJ),[1] castanospermine (CNS), endo-β-*N*-acetylglucosaminidase H (Endo H), *Streptomyces griseus* protease type XIV, and Jack bean α-mannosidase were from Sigma.

Cells and Membranes. T. cruzi epimastigote cells (Tulahuen strain, Tul 2 stock) were grown as described before (Engel & Parodi, 1985). Microsomal membranes were prepared as described by de la Canal and Parodi (1987). The preparation was dialyzed against 40 mM sodium phosphate buffer, pH 7.2, for the glucosidase II and endomannosidase assays. For continuous labeling, epimastigotes were grown in plastic flasks containing 5 mL of medium, each with the addition of 50 μCi of [^{14}C]glucose/mL and the amounts of glucosidase II inhibitors indicated in text. Pools of two flasks were used in each experiment.

Effect of DNJ and CNS on Cellular Metabolism (Short Incubations). T. cruzi cells (1 g) from the late exponential phase were washed thrice with 30 mL of the labeling solution described by Engel and Parodi (1985) and resuspended in 80 mL of the same solution. Each tube in the assay contained 1 mL of the suspension, 2.5 μCi of [^{14}C]glucose (0.01 mM final concentration), and the amounts of DNJ or CNS required

[1] Abbreviations: DNJ, deoxynojirimycin; CNS, castanospermine; Endo H, endo-β-*N*-acetylglucosaminidase H; I_{50}, concentrations of glucosidase II inhibitors required for inhibiting 50% of the enzymatic activity.

to obtain molar ratios of DNJ or CNS/glucose of 0–25. Total volume was 1.2 mL. The inhibitors were added 5 min before the glucose. After 1 or 2 min at 28 °C, incubations were stopped with 0.2 mL of 50% trichloroacetic acid. The tubes were then heated for 2 min at 100 °C and the precipitates washed twice with 1 mL of 10% trichloroacetic acid and counted. Incorporation was linear with time and was about 10000 cpm after 2 min of incubation.

Preparation of Protein-Linked Oligosaccharides. Growth of cells was stopped at the end of the exponential phase (approximately 7 days of incubation) by the addition of two volumes of methanol. After centrifugation the pellets were digested in 5 mL of 0.1 M Tris-HCl buffer, pH 8.0, 2 mM CaCl, and 2 mg of *S. griseus* protease for 48 h at 37 °C under a toluene atmosphere. The suspensions were concentrated to 1.5 mL and centrifuged. The supernatants were applied to a 1.2 × 55 cm Sephadex G-10 column equilibrated with 7% 2-propanol. 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 H (0.01 units 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 corresponded to Endo H sensitive oligosaccharides, whereas those that migrated to the cathode in the second electrophoresis contained the endo H resistant compounds.

Substrates and Standards. [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 H produced the standards $Glc_{1-3}Man_9GlcNAc$.

Standards [^{14}C]$Man_{5-9}GlcNAc$ were obtained from hen oviduct slices incubated with [^{14}C]glucose as described before (Parodi et al., 1981). $Glc_1Man_{7-9}GlcNAc$ were obtained from the labeled glycoproteins formed upon incubation of UDP-[^{14}C]Glc, urea-denatured thyroglobulin, and rat liver microsomes as described previously (Trombetta et al., 1989). Treatment of the monoglucosylated compounds with α-mannosidase produced standards $Glc_1Man_{4,5}GlcNAc$. Mannobiose, mannotriose, $Man_3GlcNAc$, and $Man_4GlcNAc$ were obtained by acetolysis of [^{14}C]$Man_{5-9}GlcNAc$. Treatment of [^{14}]$Man_{5-9}GlcNAc$ with α-mannosidase produced ManGlcNAc.

Methods. Protein was measured by the Lowry method with bovine serum albumin as standard (Lowry et al., 1951). Treatment with α-mannosidase, strong acid hydrolysis, and acetolysis were as described before (Engel & Parodi, 1985). Glucosidase II activity was measured as described previously (Bosch et al., 1988a; Ugalde et al., 1979) but with 2500 cpm of [glucose-^{14}C]$Glc_1Man_9GlcNAc$ as substrate.

Chromatography. Whatman No. 1 paper was employed. The solvents were (A) 1-propanol/nitromethane/water (5:2:4), (B) 1-butanol/pyridine/water (4:3:4), (C) 1-butanol/pyridine/water (6:4:3), and (D) 1-butanol/pyridine/water (10:3:3).

Results

The Effect of DNJ and CNS on T. cruzi Glucosidase II and on Cellular Metabolism. T. cruzi, the same as other trypanosomatids, has a glucosidase II like activity (Bosch et al., 1988a). The I_{50} values for the specific inhibitors DNJ and CNS, which are glucose analogues, were found to be 5 and 8 μM, respectively (Figure 2). These values are similar to those reported for mammalian and fungal enzymes (Elbein, 1987). The glucosidase II inhibitors did not seem to have

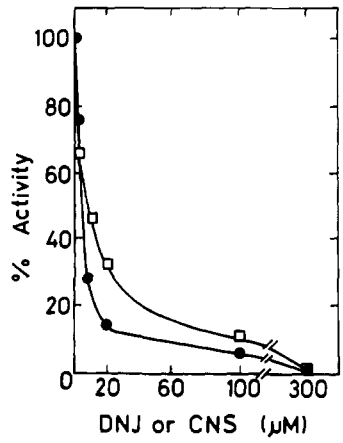

FIGURE 2: Effect of DNJ and CNS on glucosidase II activity. Glucosidase II was assayed with the indicated concentrations of DNJ (●) or CNS (□) in the incubation mixtures.

deleterious effects on cellular metabolism nor in the glucose entry into the parasite: upon incubation of *T. cruzi* cells with [^{14}]glucose and up to a 25-fold molar excess of DNJ or CNS over glucose for 1–2 min, the incorporation of label into trichloroacetic acid insoluble material (over 90% labeled amino acids in proteins) was not affected (see Experimental Procedures).

The strategy for evaluating the proportion of N-linked oligosaccharides that are glucosylated in *T. cruzi* was then to grow cells in the presence of [^{14}C]glucose and an excess of glucosidase II inhibitors and to quantitate the molar proportion of glucosylated and unglucosylated N-linked oligosaccharides.

The Effect of the Inhibitors on the Pattern of Endo H Sensitive Oligosaccharides. As mentioned above for short incubation periods, the presence of glucosidase II inhibitors did not affect cellular metabolism. The same was concluded from long incubations because no significant differences in the cell growth rate or in the incorporation of label into 66% methanol insoluble material or into N-linked oligosaccharides were observed when cells were grown in the absence or presence of 6 mM DNJ, 2.6 mM CNS, or 6 mM DNJ plus 2.6 mM CNS. The initial glucose concentration was 1 mM, that is, more than 8-fold lower than that of DNJ plus CNS in the experiment where both inhibitors were employed.

The Endo H sensitive oligosaccharides from whole cell glycoproteins were obtained as described under Experimental Procedures and run on paper chromatography. Compounds that migrated as $Man_{5-9}GlcNAc$ standards were obtained in the absence of the inhibitors (Figure 3A). In addition to those compounds, oligosaccharides that migrated as standards $Glc_1Man_{7-9}GlcNAc$ were observed when cells had been grown in the presence of 6 mM DNJ, 2.6 mM CNS, or 6 mM DNJ plus 2.6 mM CNS. Results obtained under the first condition are depicted in Figure 3B but essentially the same pattern was obtained under the other conditions. In no case were compounds migrating as $Glc_3Man_9GlcNAc$ or $Glc_2Man_9GlcNAc$ standards observed.

Evaluation of the Proportion of Glucosylated Oligosaccharides. The Endo H sensitive compounds were degraded with Jack bean α-mannosidase and the products run on paper chromatography. The products have been identified before (Parodi et al., 1983a) and were mannose and ManGlcNAc when cells had been grown in the absence of the inhibitors (Figure 4A) and mannose, ManGlcNAc, $Glc_1Man_4GlcNAc$, and $Glc_1Man_5GlcNAc$ when they had been grown in their presence (Figure 4B). In order to obtain a better separation between mannose and ManGlcNAc, both compounds were

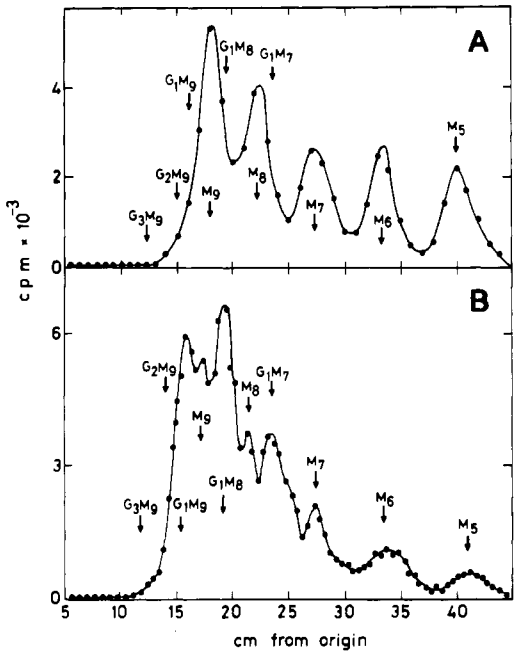

FIGURE 3: Pattern of Endo H sensitive oligosaccharides. The Endo H sensitive oligosaccharides were obtained from cells grown in the absence (A) or in the presence (B) of 6 mM DNJ and run on paper chromatography with solvent A. Standards: M_5, $Man_5GlcNAc$; M_6, $Man_6GlcNAc$; M_7, $Man_7GlcNAc$; M_8, $Man_8GlcNAc$; M_9, $Man_9GlcNAc$; G_1M_7, $Glc_1Man_7GlcNAc$; G_1M_8, $Glc_1Man_8GlcNAc$, G_1M_9, $Glc_1Man_9GlcNAc$; G_2M_9, $Glc_2Man_9GlcNAc$; G_3M_9, $Glc_3Man_9GlcNAc$.

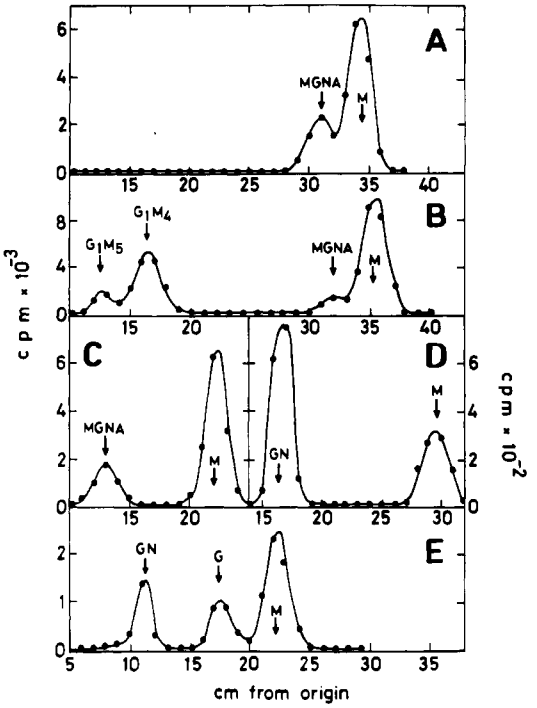

FIGURE 4: Evaluation of the proportion of glucosylated oligosaccharides. Substances in Figure 3 were treated with α-mannosidase and run on paper chromatography with solvent B (parts A and B, respectively). Substances migrating as ManGlcNAc and mannose in (A) were eluted and run on paper chromatography with solvent C (C). Substances migrating as ManGlcNAc in (C) were submitted to strong acid hydrolysis and run on paper chromatography with solvent D (D). Substances migrating as the $Glc_1Man_4GlcNAc$ standard in (B) were submitted to strong acid hydrolysis and to paper chromatography with solvent D (E). Standards: M, mannose; G, glucose, GN, glucosamine, MGNA, ManGlcNAc; G_1M_4, $Glc_1Man_4GlcNAc$; G_1M_5, $Glc_1Man_5GlcNAc$.

eluted and run with a different solvent system (Figure 4C). Elution of the disaccharide followed by strong acid hydrolysis yielded mannose and glucosamine (Figure 4D). The value of counts per minute in the mannose residues of the disaccharide (which will be referred to as *X*) is indicative of the molar amounts of unglucosylated oligosaccharides (see below). Strong acid hydrolysis of $Glc_1Man_4GlcNAc$ and $Glc_1Man_5GlcNAc$ produced labeled glucose, glucosamine, and mannose residues (Figure 4E). Label in the mannose residues in $Glc_1Man_4GlcNAc$ divided by four plus that in $Glc_1Man_5GlcNAc$ divided by five (which will be referred to as *Y*) is indicative of the molar amounts of glucosylated oligosaccharides (see below). Values were corrected according to the recovery of total radioactivity in each step of the processing of the samples. As cells were grown in the presence of [^{14}C]glucose, it may be assumed that all mannose residues have the same specific activity.

The Endo H Resistant Oligosaccharides. Although it has been reported that Endo H resistant compounds contain in *T. cruzi* N-acetylglucosamine, mannose, galactose, and sialic acid residues, no thorough structural study has been reported yet (Couto et al., 1987, 1990). On the other hand, the main Endo H resistant oligosaccharide present in glycoproteins of a different species belonging to the same genus (*Trypanosoma brucei*) has been shown to have the typical biantennary, complex-type structure (Bangs et al., 1988).

The Endo H resistant material obtained under our experimental conditions produced, upon strong acid hydrolysis, glucosamine, galactose, and mannose residues. The possible presence of sialic acid was not investigated. No glucose residues were detected even when cells had been grown in the presence of glucosidase II inhibitors (Figure 5A,B). The same pattern and no loss of radioactivity were observed when the Endo H resistant material was submitted to β-elimination (30 mM NaOH for 16 h at 30 °C) before the hydrolysis, thus indicating that the mannose units were not linked to Ser/Thr residues (Figure 5C). In addition, almost all the mannose units in the Endo H resistant material were resistant to α-mannosidase degradation (Figure 5D). It was assumed, therefore, that the Endo H resistant oligosaccharides contained mainly typical complex-type compounds, that is, having three mannose residues per molecule. The value of counts per minute in the mannose units of Endo H resistant oligosaccharides divided by three will be referred to as *Z* and is indicative of the molar amounts of unglucosylated, Endo H resistant oligosaccharides (see below).

Table I: Molar Percentage of Glucosylated N-Linked Oligosaccharides[a]

addition to growth medium	Endo H sensitive		Endo H resistant
	glucosylated	unglucosylated	unglucosylated
		82 (100)	18
6 mM DNJ	52 (59)	36 (41)	12
2.6 mM CNS	53 (62)	33 (38)	14
6 mM DNJ + 2.6 mM CNS	52 (60)	35 (40)	13

[a] Values were calculated as indicated in the text. Values in parentheses correspond to those calculated not taking into account Endo H resistant oligosaccharides.

The Molar Proportion of Glucosylated Oligosaccharides. The values of *X*, *Y*, and *Z* allowed calculation of the molar proportion of oligosaccharides that are glucosylated in the endoplasmic reticulum (Table I). It may be observed that the addition of the glucosidase II inhibitors to the growth medium only caused a partial decrease in the proportion of Endo H resistant oligosaccharides (20–30%). This might be due to the fact that not all N-linked oligosaccharides were

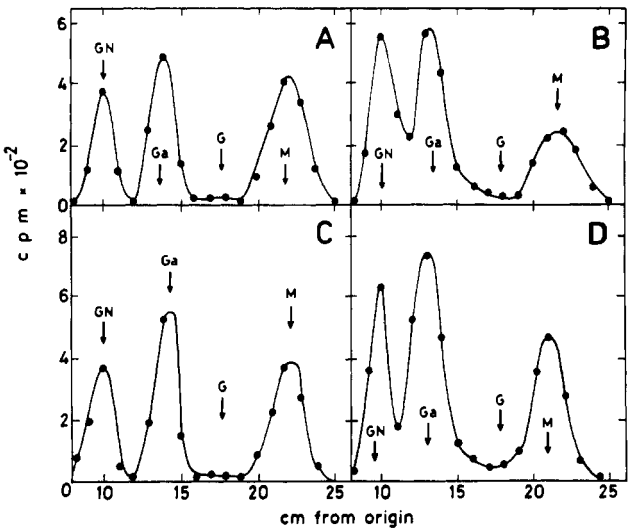

FIGURE 5: Endo H resistant oligosaccharides. The Endo H resistant glycopeptides, obtained from cells grown in the absence (A) or in the presence (B) of 6 mM DNJ, were submitted to a strong acid hydrolysis. In part C, the same glycopeptides used in part A were incubated with 0.3 mL of 30 mM NaOH for 16 h at 30 °C, neutralized with acetic acid, and run on paper electrophoresis in 10% formic acid at 25 V/cm for 120 min. Substances migrating to the cathode were submitted to strong acid hydrolysis. In part D the same glycopeptides used in parts A and C were treated with α-mannosidase and then submitted to paper electrophoresis as above. Positively charged substances were then submitted to strong acid hydrolysis. In all cases samples were run on paper chromatography with solvent D. Standards: M, mannose; G, glucose; Ga, galactose; GN, glucosamine.

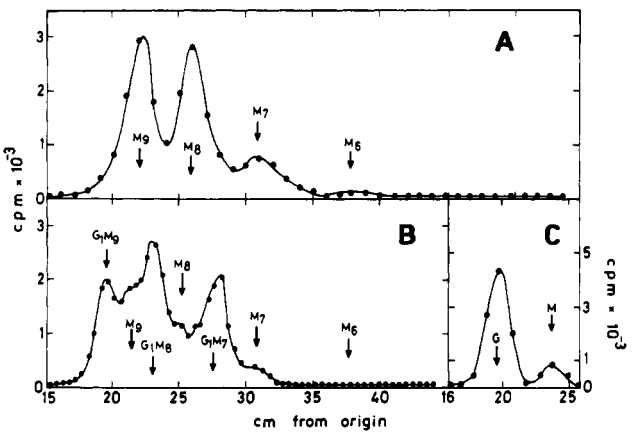

FIGURE 6: Pattern of Endo H sensitive oligosaccharides. Short incubations: (A and B) *T. cruzi* cells (1.3 g) from the late exponential phase were washed twice with 30 mL of the labeling solution described by Engel and Parodi (1985) and resuspended in 2.9 mL (total volume) of the same solution. The suspension was divided in halves and both halves were incubated with 300 μCi [^{14}C]glucose for 30 min at 28 °C in a total volume of 2 mL. The suspensions were then centrifuged for 5 min at 1000*g*. The pellets were then resuspended in 2 mL (total volume) of normal growth medium and further incubated for 30 min at 28 °C. Incubations were then stopped by the addition of 2 volumes of methanol. In part B the pulse and chase incubations contained 6 mM DNJ. This drug and [^{14}C]glucose were solubilized in the labeling solution. The Endo H sensitive oligosaccharides were obtained as described under Experimental Procedures and run on paper chromatography with solvent A. (C) Substances in part B were treated with α-mannosidase and run on paper chromatography with solvent B. Compounds migrating as the $Glc_1Man_4GlcNAc$ standard were subjected to strong acid hydrolysis followed by paper chromatography with solvent D. Standards: M_6, $Man_6GlcNAc$; M_7, $Man_7GlcNAc$; M_8, $Man_8GlcNAc$; M_9, $Man_9GlcNAc$; G_1M_7, $Glc_1Man_7GlcNAc$; G_1M_8, $Glc_1Man_8GlcNAc$; G_1M_9, $Glc_1Man_9GlcNAc$; M, mannose; G, glucose.

glucosylated and thus could be further processed to complex structures. Practically the same percentages of glucosylated oligosaccharides (52–53%) were obtained under the three different experimental conditions employed (6 mM DNJ, 2.6 mM CNS, or 6 mM DNJ plus 2.6 mM CNS) (Table I).

Short Labeling Incubations. In order to rule out the possibility that the unglucosylated compounds had been produced by deglucosylation of glucosylated oligosaccharides by unspecific glucosidases, not inhibited by DNJ or CNS and having a low affinity for the glucosylated derivatives, *T. cruzi* cells were pulsed for 30 min with [^{14}C]glucose and chased for the same period, in the absence or presence of 6 mM DNJ. The average half-life value of proteins in *T. cruzi* is unknown, but the pulse–chase period (60 min) was much shorter than the cellular doubling time (26–28 h). The pattern of Endo H sensitive oligosaccharides obtained in the absence of the inhibitor (Figure 6A) showed that the oligosaccharides had not been totally processed, as the average size of the compounds was larger than that depicted in Figure 3A. Nevertheless, unglucosylated compounds were clearly visible when the compounds obtained in the presence of 6 mM DNJ were submitted to chromatography (Figure 6B). The same methodology described above was employed to calculate the proportion of glucosylated compounds. The value obtained (63% of Endo H sensitive compounds, that is, not taking into account the Endo H resistant ones) is similar to those depicted in Table I, which were obtained from cells grown in the presence of the label and the inhibitors. It should be mentioned that the specific activity of the glucose was much higher than that of the mannose residues after the 60-min period. For instance, the hydrolysis of $Glc_1Man_4GlcNAc$ (obtained upon α-mannosidase degradation of compounds depicted in Figure 6B) gave the pattern shown in Figure 6C, which must be compared with that of Figure 4E.

Absence of an Endomannosidase. Lubas and Spiro (1987, 1988) have reported the presence of an endomannosidase in the Golgi apparatus of rat liver cells that produces GlcMan from $Glc_1Man_9GlcNAc$ and that is not inhibited by DNJ or CNS. We have been unable to detect a similar activity in *T. cruzi* microsomes: upon incubation of [glucose-^{14}C]-$Glc_1Man_9GlcNAc$ with the parasite membranes only labeled glucose was obtained. No compounds migrating as disaccharides on paper chromatography were observed even when the incubation was performed in the presence of 300 μM DNJ (Figure 7A).

Experiments performed with intact cells also discarded the possibility that the unglucosylated compounds could have been produced, in the presence of glucosidase II inhibitors by an endomannosidase-like activity. Such an enzyme would have produced $Man_{5-8}GlcNAc$ but not $Man_9GlcNAc$. Nevertheless, when the compounds migrating as the $Man_9GlcNAc$ standard in Figure 3B (that is, between 17.0 and 17.5 cm) were treated with α-mannosidase, among other products yielded by the adjacent glucosylated compounds, the presence of ManGlcNAc was observed (Figure 7B). This confirmed the presence of $Man_9GlcNAc$ in the sample.

Moreover, the action of an endomannosidase on the glucosylated compounds would have produced $Man_8GlcNAc$ and $Man_7GlcNAc$ isomers in which the *N*-acetylglucosamine-bearing fragment obtained upon acetolysis of the compounds should have the composition $Man_3GlcNAc$. Acetolysis is a chemical reaction that preferentially cleaves α(1,6) bonds between mannose residues. Substances migrating as $Man_8GlcNAc$ and $Man_7GlcNAc$ standards in Figure 3B were eluted and subjected to a second chromatography in order to eliminate adjacent compounds and then to acetolysis. As shown in Figure 7C (acetolysis of $Man_7GlcNAc$), $Man_4GlcNAc$ and not $Man_3GlcNAc$ was the main *N*-

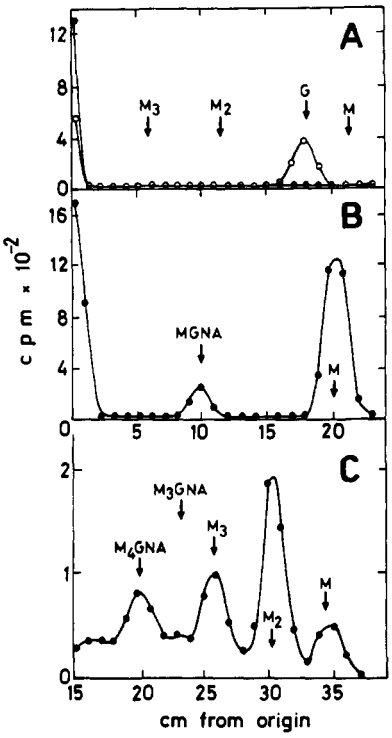

FIGURE 7: Absence of an endomannosidase. (A) *T. cruzi* membranes were incubated with [glucose-^{14}C]$Glc_1Man_9GlcNAc$ under the conditions used for the glucosidase II assay in the absence (○) or presence (●) of 0.3 mM DNJ. The incubations were stopped by the addition of 1 mL of water followed by heating at 100 °C for 5 min. The supernatants were desalted with an Amberlite (H^+ and acetate forms) resin and run on paper chromatography with solvent C. (B) Substances migrating as a $Man_9GlcNAc$ standard (17.0–17.5 cm) in Figure 3B were treated with α-mannosidase and run on paper chromatography with solvent C. (C) Substances migrating as a $Man_7GlcNAc$ standard (26.5–28.5 cm) in Figure 3B were rerun on paper chromatography with solvent A, submitted to acetolysis, and run on paper chromatography with solvent B. Standards: M, mannose; M_2, mannobiose; M_3, mannotriose; MGNA, ManGlcNAc; M_3GNA, $Man_3GlcNAc$; M_4GNA, $Man_4GlcNAc$; G, glucose.

acetylglucosamine-containing fragment. The same result was obtained upon acetolysis of $Man_8GlcNAc$.

Discussion

The choice of *T. cruzi* cells for evaluating the proportion of N-linked oligosaccharides that are glucosylated proved to have several advantages: (a) Unlike what happens in all wild-type mammalian, plant, and fungal cells, the addition of glucose residues to proteins occurs in *T. cruzi* cells (as in all trypanosomatids) solely by the action of the UDP-Glc:glycoprotein glucosyltransferase because unglucosylated oligosaccharides are transferred to proteins in this parasite. In all other eukaryotic cells it is almost impossible to distinguish monoglucosylated protein-linked oligosaccharides derived from the oligosaccharide transferred in protein N-glycosylation from those produced by reglucosylation of glucose-free compounds. Certain *Saccharomyces cerevisiae* mutants defective in the synthesis of dolichol-P-Glc or in the transfer of glucosyl residues from the last compound to dolichol-P-P derivatives also transfer, the same as trypanosomatids, unglucosylated oligosaccharides in protein N-glycosylation (Ballou et al., 1986; Runge & Robbins, 1986; Runge et al., 1984). However, due to the fact that the yeast (as well as mammalian and plant) oligosaccharyltransferase requires the presence of three glucose residues in the oligosaccharide in order to catalyze an efficient transfer reaction, glycoproteins in the above-mentioned mutants are heavily underglycosylated (Ballou et al., 1986). This makes those mutants unfit for the purpose of this work. (a) The trypanosomatid oligosaccharyltransferase, on the other hand, does not require the presence of glucosyl units in the oligosaccharide (Bosch et al., 1988a). (b) As shown above, *T. cruzi* cells grow at the same rate in the presence or absence of glucosidase II inhibitors. No effect of DNJ or CNS (which are glucose analogues) on the incorporation of label from the precursor ([^{14}C]glucose) into 66% methanol-insoluble material or into N-linked oligosaccharides was observed. (c) No evidence for the presence in *T. cruzi* of an endomannosidase similar to that described by Lubas and Spiro (1987, 1988) in a mammalian tissue was obtained in experiments performed either with intact cells or with cell-free preparations. As the endomannosidase has been reported not to be inhibited by DNJ or CNS, its presence in *T. cruzi* would have produced unglucosylated compounds from the glucosylated ones.

Cell-free assays showed that the I_{50} values of *T. cruzi* glucosidase II for DNJ and CNS (5 and 8 μM, respectively) are comparable to those observed before for the fungal or mammalian enzymes. The concentrations of inhibitors in the growth medium were, therefore, 300–1500-fold higher than the I_{50} values. It is unknown whether the concentrations found in the lumen of the endoplasmic reticulum (where presumably glucosidase II is located) are similar to those of the external milieu, but it has been determined that deoxymannojirimycin (and probably DNJ) does not penetrate into mammalian cells through the hexose transporter but penetrates through facilitated diffusion (Neefjes et al., 1989). The fact that a 20–25-fold molar excess of DNJ or CNS over that of glucose did not interfere with the incorporation of label into trichloroacetic acid insoluble material suggests that the inhibitors and glucose penetrate into *T. cruzi* cells by different mechanisms. Whether the former compounds penetrate through facilitated diffusion as in mammalian cells is an open question.

Results presented here shown that fully 52–53% of N-linked oligosaccharides were glucosylated in *T. cruzi* cells. The presence of unglucosylated oligosaccharides were apparently not due to the action of unspecific glucosidases not inhibited by DNJ or CNS that could have acted at a slow rate on the glucosylated compounds, because the same percentages of glucosylated and unglucosylated Endo H sensitive saccharides were obtained in cells grown in the presence of inhibitors or in cells incubated for only 60 min with them.

The fact that not all oligosaccharides were glucosylated might be related to their different accessibility to the UDP-Glc:glycoprotein glucosyltransferase. As mentioned above, native thyroglobulin was not glucosylated in in vitro assays by the rat liver enzyme, whereas the denatured glycoprotein was (Trombetta et al., 1989). Alternatively, for unknown reasons, totally inhibitory concentrations of DNJ or CNS might not be attained in the lumen of the endoplasmic reticulum.

On the average, glycoproteins have more than one N-linked oligosaccharide per molecule. Therefore, the actual percentage of glycoproteins that are glucosylated must be higher than 52–53%, but it is highly unlikely that all glycoproteins are glucosylated. We have recently determined that in a cysteine proteinase from *T. cruzi* (a lysosomal enzyme having three oligosaccharides per molecule), only 30–35% of the oligosaccharides were glucosylated (unpublished results). This suggests that only one of the oligosaccharides might be glucosylated. The high proportion of N-glycoproteins that are normally glucosylated argues against the interpretation proposed by Suh et al. (1989) of their own results (see the introduction).

Data reported here were obtained by using *T. cruzi* cells. It is highly improbable that exactly the same percentage of N-linked oligosaccharides is glucosylated in other eukaryotic cells. Nevertheless, results obtained strongly suggest that in all cells direct glucosylation of glycoproteins in the endoplasmic reticulum affects a major proportion of N-linked glycoproteins.

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