

Retention of Glucose Units Added by the UDP-Glc:Glycoprotein Glucosyltransferase Delays Exit of Glycoproteins from the Endoplasmic Reticulum

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Abstract. It has been proposed that the UDP-Glc:glycoprotein glucosyltransferase, an endoplasmic reticulum enzyme that only glucosylates improperly folded glycoproteins forming protein-linked $\text{Glc}_1\text{Man}_{7-9}\text{-GlcNAc}_2$ from the corresponding unglucosylated species, participates together with lectin-like chaperones that recognize monoglucosylated oligosaccharides in the control mechanism by which cells only allow passage of properly folded glycoproteins to the Golgi apparatus. *Trypanosoma cruzi* cells were used to test this model as in trypanosomatids addition of glucosidase inhibitors leads to the accumulation of only monoglucosylated oligosaccharides, their formation being catalyzed by the UDP-Glc:glycoprotein glucosyltransferase. In all other eukaryotic cells the inhibitors produce underglycosylation of proteins and/or accumulation of oligosaccharides containing two or three glucose units. Cruzipain, a lysosomal proteinase having

three potential N-glycosylation sites, two at the catalytic domain and one at the COOH-terminal domain, was isolated in a glucosylated form from cells grown in the presence of the glucosidase II inhibitor 1-deoxy-nojirimycin. The oligosaccharides present at the single glycosylation site of the COOH-terminal domain were glucosylated in some cruzipain molecules but not in others, this result being consistent with an asynchronous folding of glycoproteins in the endoplasmic reticulum. In spite of not affecting cell growth rate or the cellular general metabolism in short and long term incubations, 1-deoxynojirimycin caused a marked delay in the arrival of cruzipain to lysosomes. These results are compatible with the model proposed by which monoglucosylated glycoproteins may be transiently retained in the endoplasmic reticulum by lectin-like anchors recognizing monoglucosylated oligosaccharides.

TRANSFER of the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ from a dolichol-P-P derivative to nascent polypeptide chains is followed by the immediate removal of the glucose units. Two glucosidases have been described to be involved in this process: glucosidase I, an $\alpha(1, 2)$ glucosidase that removes the more external residues and glucosidase II, an $\alpha(1,3)$ glucosidase responsible for removal of the other two units. All reactions mentioned above have been described to occur in the lumen of the endoplasmic reticulum. One or two mannose units may be cleaved in the same subcellular location (Fig. 1 a) (21).

An additional processing reaction also occurring in the endoplasmic reticulum is the transient glucosylation of high mannose-type, protein-linked oligosaccharides: as described to occur in mammalian cells $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2$, $\text{Glc}_1\text{Man}_8\text{GlcNAc}_2$ and $\text{Glc}_1\text{Man}_7\text{GlcNAc}_2$ are formed from the corresponding unglucosylated compounds (Fig. 1 a) (32, 33). The newly added glucose units are immediately removed by glucosidase II, as the glucoses are linked to the

same mannose and with the same bond as in $\text{Glc}_1\text{Man}_9\text{GlcNAc}_2\text{-P-P-dolichol}$. The glucosylating enzyme (UDP-Glc:glycoprotein glucosyltransferase) has been detected in mammalian, plant, fungal, and protozoan cells (38). The enzyme appeared to be a soluble protein of the lumen of the endoplasmic reticulum and to have a remarkable property: it glucosylated in cell-free assays denatured glycoproteins whereas native species or glycopeptides were not glucosylated (12, 36, 38–40). Recognition by the glucosyltransferase of a protein domain only exposed in denatured conformations was required for the transfer reaction (36). The glucosyltransferase behaved, therefore, as a sensor of unfolded, partially folded and misfolded conformations.

Proteins entering the secretory pathway acquire their final tertiary and in some cases also quaternary structures in the lumen of the endoplasmic reticulum. Species that fail to fold properly are retained in that subcellular location where they are proteolytically degraded (24). A very stringent quality control is therefore required to prevent passage of misfolded proteins to the Golgi cisternae. A model for such quality control applicable to glycoproteins has been recently proposed (16, 18). According to it, high mannose-type oligosaccharides in the endoplasmic reticu-

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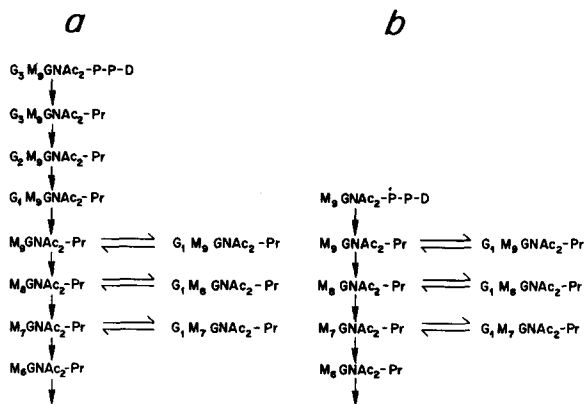


Figure 1. Initial glycoprotein processing reactions occurring in mammalian (a) and *T. cruzi* (b) cells. *G* stands for glucose; *M* for mannose; *GNAC* for N-acetylglucosamine; *D* for dolichol; and *Pr* for protein.

lum shuttle between monoglucosylated and unglucosylated structures, their formation catalyzed by the UDP-Glc:glycoprotein glucosyltransferase and glucosidase II. A membrane-bound chaperone, calnexin, that has a lectin-like activity that recognizes the monoglucosylated oligosaccharides (16), would bind the monoglucosylated structures, and thus retain glycoproteins in the endoplasmic reticulum as long as the protein moieties are not properly folded. On attaining the correct native conformations, glycoproteins would become substrates for the glucosidase but not for the glucosyltransferase and thus be liberated from the calnexin anchor. Glycoproteins would then be able to be transported to the Golgi apparatus.

This model predicts that inhibition of removal of the glucose units added by the glucosyltransferase would delay or abolish (depending on how tightly the lectin binds monoglucosylated oligosaccharides) exit of glycoproteins from the endoplasmic reticulum. This prediction cannot be tested, however, in most eukaryotic cells as glucosidase II is responsible for removal of both $\alpha(1,3)$ -linked glucose units. Moreover, known inhibitors of glucosidase II also inhibit glucosidase I (21). Addition of those drugs to cells leads to the accumulation of oligosaccharides having two or three glucoses that are not recognized by calnexin (16, 17, 20).

The prediction can be tested, however, in trypanosomatid protozoa. These parasites are the only known wild-type cells in which unglucosylated oligosaccharides are transferred in vivo to nascent polypeptide chains and, therefore, only monoglucosylated oligosaccharides are formed in them (28). Initial glycoprotein processing reactions occurring in *Trypanosoma cruzi* cells are depicted in Fig. 1 *b* (31). The main drawback encountered with trypanosomatids when studying intracellular transit of glycoproteins is that there are almost no known glycoproteins that fulfill two basic requisites for this study, that is, to be synthesized in sufficient amounts to allow checking if in vivo they are indeed glucosylated, and to be soluble secreted or lysosomal glycoproteins to allow testing the effect of retention of glucose units on the time required for arrival to their final destinations.

In this paper, we describe the effect of inhibiting re-

moval of glucose units exclusively added by the UDP-Glc:glycoprotein glucosyltransferase on the time required by newly synthesized cruzipain, a cysteine proteinase having high mannose-type oligosaccharides, to reach the lysosomes (2, 8). This is practically the only known trypanosomatid glycoprotein that fulfills the above mentioned requisites. It is currently accepted that differences in the time required for arrival to their final destination among glycoproteins having the same localization (external milieu, plasma membrane, lysosomes, etc.) reflect differences in the time required for leaving the endoplasmic reticulum (24).

Materials and Methods

Materials

Jack bean α -mannosidase, 1-deoxynojirimycin (DNJ),¹ monosaccharide standards, bovine thyroglobulin, *p*-nitrophenyl- α -D-mannoside, endo- β -N-acetylglucosaminidase H (Endo H), *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64) and *Streptomyces griseus* protease type XIV (Pronase) were from Sigma Chem. Co. (St. Louis, MO). Ham's F-12 medium (methionine, proline, and glycine free) was purchased from Biochrom KG (Berlin, Germany). [¹⁴C]Glucose (320 Ci/mol) was from ARC (St. Louis, MO) and [³⁵S]methionine was from New England Nuclear (Wilmington, DE). Con A-Sepharose and Sephadex G-25 prepacked (NAP 10) columns were from Pharmacia (Uppsala, Sweden).

Standards

[¹⁴C]Man_{6,9}GlcNAc standards from hen oviduct and [¹⁴C]Glc₁Man_{7,9}GlcNAc from rat liver microsomes were prepared as described previously (30, 38). Treatment of [¹⁴C]Man_{6,9}GlcNAc with α -mannosidase produced ManGlcNAc and that of Glc₁Man_{7,9}GlcNAc with the same enzyme yielded Glc₁Man_{4,5}GlcNAc.

Cells

T. cruzi cells of the Tulahuen strain (Tul 2 stock) were grown as described before (7). For the purification of cruzipain, cells from a 20-ml culture containing 1–2 mCi of [¹⁴C]glucose and where indicated also 6 mM DNJ were harvested at a density of $4\text{--}6 \times 10^7$ cells/ml and mixed with those obtained from an unlabeled culture.

Purification and Self-Proteolysis of Cruzipain

The proteinase was purified to homogeneity from the mixture of labeled and unlabeled cells as previously described (6). It was submitted to self-proteolysis as before (19).

Preparation of the COOH-Terminal Domain and of Glycopeptides from the Catalytic Domain

The autolysis mixture was dialyzed against 5 mM triethylamine-acetate buffer, pH 7.2. The dialysate was lyophilized and the glycopeptides arising from the catalytic domain were separated from amino acids and small peptides by gel filtration chromatography through a 57×1.2 cm Sephadex G-10 column equilibrated against 7% 2-propanol. The COOH-terminal domain of cruzipain was separated from the undegraded enzyme by gel filtration through a Superose 12 column in a FPLC system as described before (9).

Preparation of Endo H-sensitive Oligosaccharides from the COOH-Terminal Domain

The COOH-terminal domain was incubated overnight at 37°C in 1 ml of 0.15 M Tris-HCl buffer, pH 8.0, 5 mM CaCl₂ and 15.0 mg of *S. griseus* protease (Pronase). The solution was desalted through a 1.2×57 cm Sephadex G-10 column equilibrated with 7% 2-propanol. Material in the void volume was submitted to paper electrophoresis in 10% formic acid for 3 h

1. Abbreviations used in this paper: DNJ, 1-deoxynojirimycin; Endo H, endo- β -N-acetylglucosaminidase H.

at 26 V/cm. Positively charged substances that migrated 8–20 cm to the cathode were eluted and treated with Endo H (0.01 U in 0.3 ml of 75 mM triethylamine-acetate buffer, pH 5.5, for 16 h at 37°C). The samples were then submitted to paper electrophoresis as above. Neutral substances represented the sensitive compounds.

Preparation of Endo H-sensitive Oligosaccharides from the Catalytic Domain

The glycopeptides from the catalytic domain obtained as described above were treated with Endo H and the sensitive oligosaccharides isolated as performed for the oligosaccharides from the COOH-terminal domain.

Preparation of Oligosaccharides from the Whole Enzyme

Endo H-sensitive oligosaccharides from pure cruzipain were prepared as described above for those obtained from the COOH-terminal domain.

Subcellular Fractionation and Cell Disruption by Freezing and Thawing

Cells were ground in a mortar with silicon carbide and submitted to differential centrifugation as described before (2). For cell disruption, by freezing and thawing, cells were twice washed with 0.25 M sucrose, 5 mM KCl and the pellet obtained upon a low speed centrifugation was kept frozen at -20°C for 48 h, after which cells were thawed and resuspended in 0.1 M sodium phosphate buffer, pH 7.1, 0.15 M NaCl. The suspension was centrifuged for 10 min at 15,000 *g*. The supernatant was removed and the pellet was resuspended in phosphate-buffered saline and sonicated. The suspension was centrifuged as above.

Pulse Labeling of Cells with [^{14}C]Glucose

T. cruzi cells (1 g) from the late exponential phase were washed three times with 30 ml of the labeling solution described previously (11) and resuspended in 80 ml of the same solution. Each tube in the assay contained 1 ml of the suspension, 2.5 mCi of [^{14}C]glucose (0.01 mM final concentration) and the amounts of DNJ required to obtain molar ratios of DNJ/glucose of 0–25. Total volume was 1.2 ml. The inhibitor was added 5 min before the glucose. After 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 10% trichloroacetic acid and counted.

Pulse-chase Labeling of *T. cruzi* Cells with [^{35}S]Methionine

Cells in the exponential phase (4.0×10^7 cells/ml) were harvested and 2.5 g of them were twice washed with Ham's F-12 (methionine, proline, and glycine free) medium (10.65 g per liter) supplemented with 34.5 mg per liter of proline and 7.5 mg per liter of glycine and 1.2 g per liter of NaHCO_3 .

The parasites were resuspended in 9 ml of the above indicated medium. The suspension was divided in halves. DNJ was added to one of them up to 6 mM final concentration. After 20 min at 28°C, 2 mCi of [^{35}S]methionine were added and both aliquots were incubated for 15 min at 28°C. The suspensions were submitted to low speed centrifugations and the pellets were washed with 5 ml of *T. cruzi* normal growth medium (7) supplemented with 3 mM methionine. DNJ (6 mM) was added to the medium used for washing cells incubated with the drug. Pellets were resuspended in 5 ml of the respective washing media and aliquots of 0.25 ml were withdrawn after 0, 5, 10, 20, 30, 50, 100, 150, 200, and 300 min at 28°C. The suspensions were centrifuged and the pellets were frozen for 48 h at -20°C . The pellets were resuspended in 1 ml of 50 mM Tris-HCl buffer, pH 7.6, 0.15 M NaCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 2 mM MnCl_2 and 0.1 mM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), the suspensions were then centrifuged for 10 min at 15,000 *g* and the supernatants applied to 0.8 ml Con A-Sepharose columns. The columns were washed with the same buffer until no labeled substances were eluted. Cruzipain was eluted with 1 ml of the same buffer containing 0.5 M α -methylmannoside. The samples were desalted with Sephadex G-25 prepacked columns and concentrated in a Speed-Vac equipment. Samples were then submitted to SDS-containing 10% polyacrylamide gel electrophoresis and to autoradiography. Cruzipain-containing portions of the gels were sliced and counted. In the case of experiment shown in Fig. 4 c, freeze-thawed

cells were resuspended in buffer and centrifuged. The supernatants were withdrawn and the pellets were resuspended in buffer, sonicated, and centrifuged again. The first and second supernatants were processed as above.

Enzymatic Assays

Cruzipain, α -mannosidase, glucosidase II, and UDP-Glc:glycoprotein glucosyltransferase were assayed as previously described (5, 23, 38, 41). For glucosidase II [^{14}C]Glc₁Man_{7,9}GlcNAc was used as substrate whereas for the glucosyltransferase UDP-[^{14}C]Glc and 8 M urea-denatured thyroglobulin were employed.

Methods

Strong acid hydrolysis was performed in 1 M HCl at 100°C for 4 h. The samples were applied to a Dowex 1 (acetate form) Pasteur pipette column after hydrolysis. Chromatography was performed on Whatman 1 papers. The following solvents were used: A, 1-propanol/nitromethane/water (5:2:4); B, 1-butanol/pyridine/water (4:3:4); and C, 1-butanol/pyridine/water (10:3:3). Degradation with α -mannosidase was as described before (11).

Results

Cruzipain Is Glucosylated In Vivo by the UDP-Glc: Glycoprotein Glucosyltransferase

T. cruzi cells were grown in the presence or absence of 6 mM DNJ in a medium containing [^{14}C]glucose. Labeled cells were mixed with unlabeled ones and cruzipain was purified to homogeneity. The cysteine proteinase was then degraded with an unspecific proteinase (Pronase) and resulting glycopeptides were treated with Endo H. Oligosaccharides thus liberated were run on paper chromatography. The sample isolated from cells grown in the presence of the glucosidase II inhibitor showed peaks or shoulders in the position of Glc₁Man₉GlcNAc, Man₉GlcNAc, Glc₁Man₈GlcNAc, Man₈GlcNAc, Glc₁Man₇GlcNAc, Man₇GlcNAc, and Man₆GlcNAc standards (Fig. 2 a) whereas cruzipain purified from cells grown in the absence of DNJ only showed peaks in the position of the unglucosylated standards (Fig. 2 b).

Oligosaccharides Linked to the Same Asparagine Residue Are Glucosylated in Some Cruzipain Molecules but Not in Others

Cruzipain has three potential N-glycosylation sites, two in the catalytic domain and one in the COOH-terminal domain (4). Of both sites in the catalytic domain, that closer to the NH_2 terminus is glycosylated whereas it is unknown whether the middle one is indeed occupied (unpublished results). On the other hand, it has been already established that the site at the COOH-terminal domain is glycosylated (9).

As shown in Fig. 2 a, both glucosylated and unglucosylated oligosaccharides were present in cruzipain molecules isolated from cells grown in the presence of DNJ. Two possibilities may be envisaged: either only oligosaccharides at some glycosylation sites were glucosylated or, alternatively, oligosaccharides at the same glycosylation site were glucosylated in some cruzipain molecules but not in others. Oligosaccharides present at the COOH-terminal and catalytic domains can be easily separated because on self proteolysis cruzipain produces a COOH-terminal domain with an apparent molecular weight of 25 kD. The COOH-terminal domain can be separated from glycopep-

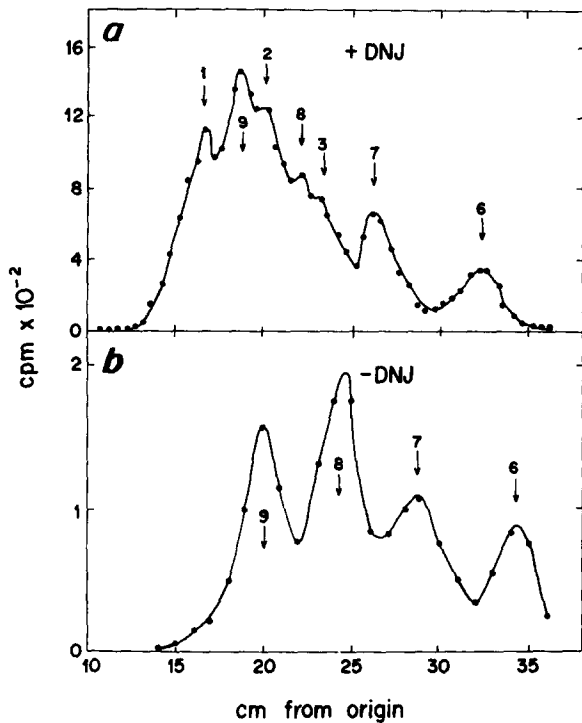


Figure 2. Cruzipain oligosaccharides. The endo H-sensitive oligosaccharides were isolated from cruzipain purified from cells grown in the presence of [^{14}C]glucose and DNJ (a), or in the absence of the inhibitor (b) and run on paper chromatography in solvent A. For further details see Materials and Methods. Standards: 1, $\text{Glc}_1\text{Man}_9\text{GlcNAc}$; 2, $\text{Glc}_1\text{Man}_8\text{GlcNAc}$; 3, $\text{Glc}_1\text{Man}_7\text{GlcNAc}$; 9, $\text{Man}_9\text{GlcNAc}$; 8, $\text{Man}_8\text{GlcNAc}$; 7, $\text{Man}_7\text{GlcNAc}$; and 6, $\text{Man}_5\text{GlcNAc}$.

tides generated from the NH_2 -terminal domain by dialysis and from undegraded cruzipain by gel filtration chromatography.

The oligosaccharides at the single COOH-terminal domain glycosylation site were isolated as described above for the whole cruzipain molecule. They were degraded with α -mannosidase and run on paper chromatography. As shown in Fig. 3 a, substances migrating as mannose, ManGlcNAc , $\text{Glc}_1\text{Man}_4\text{GlcNAc}$, and $\text{Glc}_1\text{Man}_5\text{GlcNAc}$ standards appeared as degradation products. The oligosaccharides at the NH_2 -terminal domain gave a similar pattern (Fig. 3 b). Chromatography of substances migrating as mannose and ManGlcNAc standards in Fig. 3 a in a different solvent system confirmed the presence of mannose and ManGlcNAc among the degradation products (Fig. 3 c). On the other hand, strong acid hydrolysis of the substance migrating as the $\text{Glc}_1\text{Man}_4\text{GlcNAc}$ standard in Fig. 3 a confirmed that it was composed by N-acetylglucosamine, glucose, and mannose residues (Fig. 3 d). Treatment of oligosaccharides isolated from cruzipain purified from cells grown in the absence of DNJ (Fig. 2 b) with α -mannosidase only produced mannose and ManGlcNAc (not shown).

The presence of both the disaccharide ManGlcNAc and the hexasaccharide $\text{Glc}_1\text{Man}_4\text{GlcNAc}$ among the α -mannosidase degradation products of the oligosaccharides located at the COOH-terminal domain indicated that both glucosylated and unglucosylated species were present at

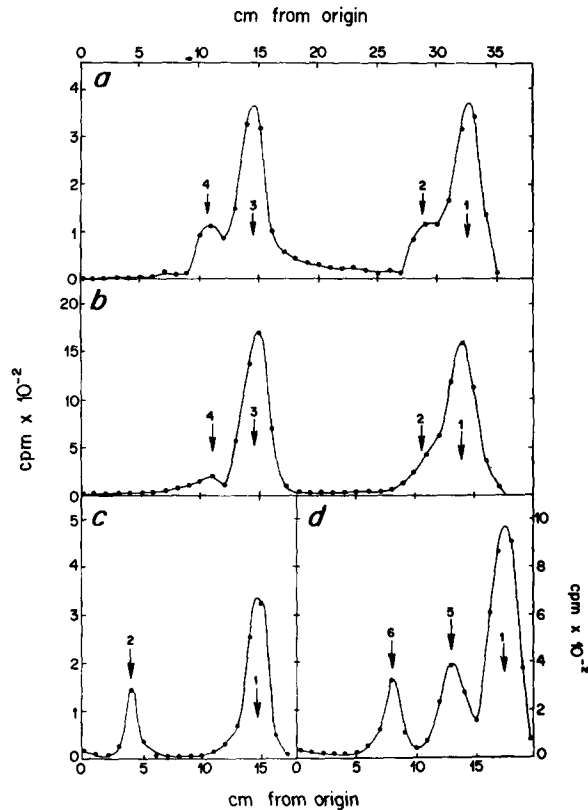


Figure 3. Characterization of cruzipain oligosaccharides. The oligosaccharides from the COOH-terminal (a) or catalytic (b) domains were isolated from cruzipain purified from cells grown in the presence of [^{14}C]glucose and DNJ, treated with α -mannosidase, and run on paper chromatography in solvent B. (c) Substances migrating as standards 1 and 2 in a were eluted and run on paper chromatography in solvent C. (d) Substances migrating as standard 3 in a were submitted to strong acid hydrolysis (1 M HCl for 4 h at 100°C) and run on paper chromatography in solvent C. For further details see Materials and Methods. Standards: 1, mannose; 2, ManGlcNAc ; 3, $\text{Glc}_1\text{Man}_4\text{GlcNAc}$; 4, $\text{Glc}_1\text{Man}_5\text{GlcNAc}$; 5, glucose; and 6, glucosamine.

that site: the disaccharide (with a β -bond not cleaved by α -mannosidase) is indicative of the presence of unglucosylated oligosaccharides whereas the presence of $\text{Glc}_1\text{Man}_4\text{GlcNAc}$ indicates the presence of glucosylated compounds (the presence of the glucose unit precludes further α -mannosidase degradation, as this is an exoglycosidase).

The proportion of glucosylated species at the COOH-terminal domain oligosaccharides was found to be 64%. The same procedure employed previously for calculating the proportion of glucosylated and unglucosylated oligosaccharides in whole cell glycoproteins was used now (13).

The UDP-Glc:Glycoprotein Glucosyltransferase and Cruzipain Are Located in Different Subcellular Locations

Although it has been firmly established that in mammalian cells the UDP-Glc:glycoprotein glucosyltransferase is located in the endoplasmic reticulum (39), it was necessary to establish the subcellular localization of the enzyme in *T. cruzi* cells. For this purpose parasite cells were ground with silicon carbide in a mortar and subsequently submit-

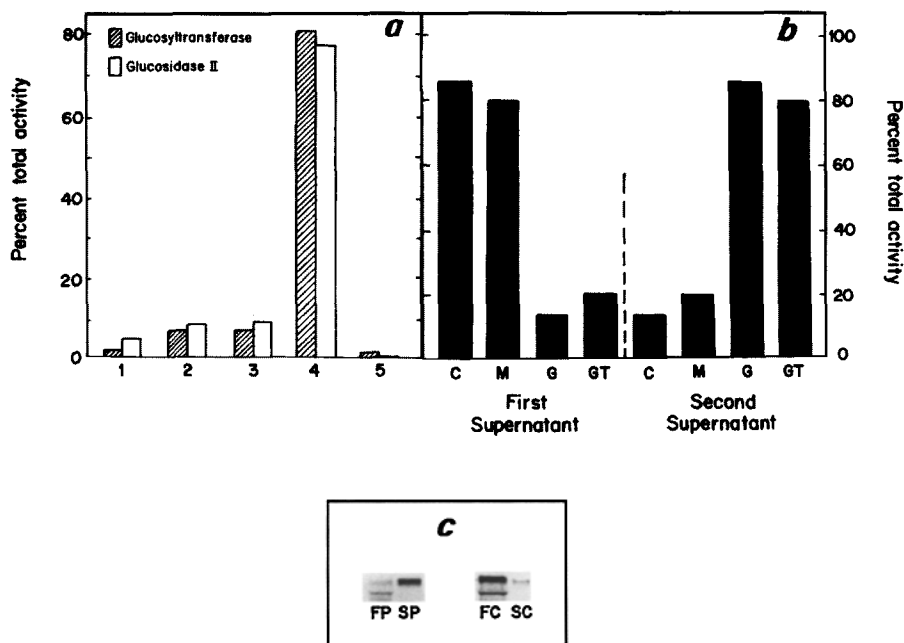


Figure 4. Subcellular distribution of enzymes. (a) *T. cruzi* cells were ground with silicon carbide in a mortar and resulting material submitted to differential centrifugation. UDP-Glc:glycoprotein glucosyltransferase and glucosidase II were assayed in the following fractions: 1, nuclear; 2, large granules; 3, small granules; 4, microsomes; and 5, soluble. (b) *T. cruzi* cells were freeze-thawed, resuspended in buffered saline and centrifuged. The supernatants were withdrawn and the pellets were resuspended in buffer, sonicated, and centrifuged again. Cruzipain (C), α -mannosidase (M), glucosidase II (G), and UDP-Glc:glycoprotein glucosyltransferase (GT) were assayed in the first and second supernatants. (c) *T. cruzi* cells were pulsed with [³⁵S]methionine for 15 min and chased with the unlabeled amino acid for 300 min. Pulse and chase samples were freeze-thawed and processed as in b. The four supernatants were submitted to Con A affinity

chromatography and material eluted with 0.5 M α -methylmannoside run on 10% polyacrylamide gel electrophoresis. FP and SP correspond to the first and second supernatants of the pulse sample and FC and SC to those of the chase one, respectively.

ted to differential centrifugation. As depicted in Fig. 4 a, both UDP-Glc:glycoprotein glucosyltransferase and glucosidase II appeared in the microsomal fraction. In mammalian cells both are soluble proteins of the lumen of the endoplasmic reticulum, although the second one appeared to be loosely attached to membranes (37, 39). Most of cruzipain appeared in the soluble fraction when exactly the same fractionation procedure was employed (2). Lysosomes are extremely fragile and most of them are ruptured when *T. cruzi* cells are ground with silicon carbide in a mortar.

Another procedure employed for assessing the differential localization of transient glucosylation of glycoproteins and cruzipain was to freeze and thaw intact *T. cruzi* cells and to assay two lysosomal enzymes, cruzipain and α -mannosidase, and both enzymes responsible for transient glucosylation of glycoproteins, the UDP-Glc:glycoprotein glucosyltransferase and glucosidase II in the supernatant of a 15,000 g for 10 min centrifugation as well as in a second supernatant obtained after resuspension, sonication, and centrifugation of the resulting pellet. As depicted in Fig. 4 b, the majority of cruzipain and α -mannosidase appeared in the first supernatant whereas the glucosyltransferase and glucosidase II appeared mainly in the second one. This method afforded, therefore, a rapid procedure for separating the soluble content of the endoplasmic reticulum from that of lysosomes and was used below for assessing arrival of newly synthesized cruzipain to lysosomes.

To confirm the reliability of the method, *T. cruzi* cells were pulsed with [³⁵S]methionine for 15 min and chased for 300 min with the unlabeled amino acid. Cells were freeze-thawed and processed as above. The four supernatants (first and second from the pulse and chase samples) were submitted to affinity chromatography in Con A-Sepharose columns. Material eluting with α -methylman-

noside was run on polyacrylamide gel electrophoresis under denaturing conditions. Loss of cruzipain in the affinity chromatography was almost nil under the experimental conditions employed. Moreover, preliminary experiments had shown that cruzipain (that constitutes ~5% of all soluble cellular proteins) is by far the main soluble protein having high mannose-type oligosaccharides. As shown in Fig. 4 c, whereas after a 15-min pulse cruzipain appeared in the second supernatant, after a 300-min chase the cysteine proteinase appeared mainly in the first one. In this case cruzipain was present as a double band, as described before for the mature enzyme (8). Results shown in Fig. 4 c confirm, therefore, the reliability of the method employed for separation of soluble proteins of the endoplasmic reticulum from those present in lysosomes and indicate that the double band is the consequence of a posttranslational modification occurring in the Golgi apparatus or in lysosomes.

DNJ Does Not Affect T. cruzi Cell Growth Rate or the Parasite General Metabolism but Partially Affects Cruzipain Synthesis and the Cruzipain Content of Lysosomes

DNJ did not noticeably affect *T. cruzi* cell growth rate when added at a concentration ~1,200-fold higher than that required for a 50% inhibition of glucosidase II (Fig. 5 a). Samples of parasites were withdrawn from cultures having different cell densities and submitted to freezing and thawing to liberate cytosolic plus lysosomal proteins. The same protein concentrations were found in the supernatants of 15,000 g for 10 min centrifugations of inhibitor-containing and inhibitor-devoid samples, thus confirming the lack of effect of DNJ on cellular general metabolism. The glucosidase II inhibitor (a glucose analog) not only

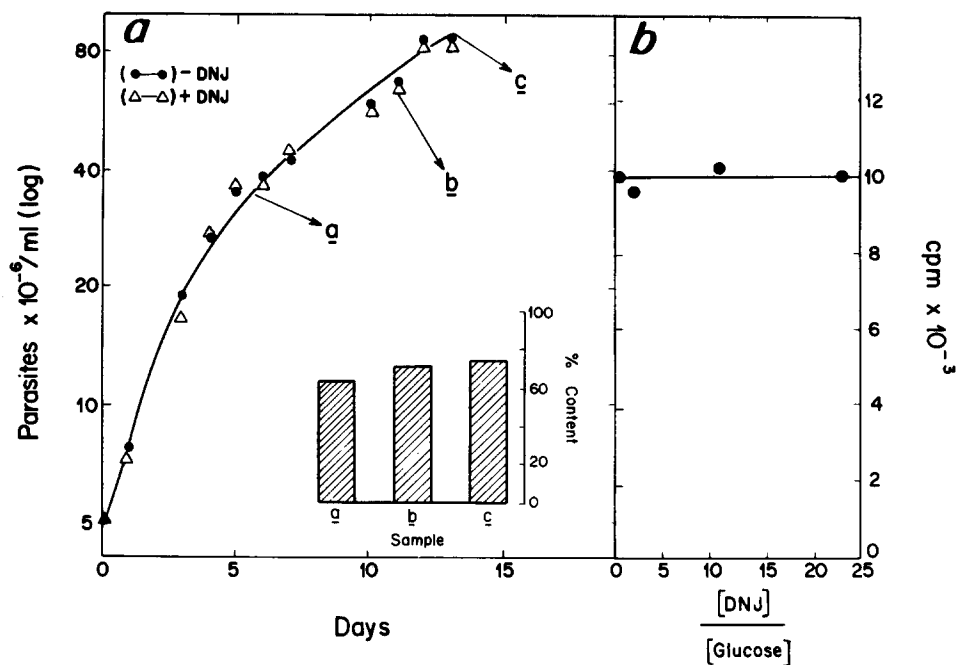


Figure 5. The effect of DNJ on cell growth, general cell metabolism, and cruzipain content. (a) *T. cruzi* cells were grown in the presence (Δ - Δ) or absence (\bullet - \bullet) of 6 mM DNJ. (a) Inset: where indicated in a (a, b and c) cells were withdrawn, submitted to freezing and thawing, and cruzipain activity assayed in the supernatants of 15,000 g for 10-min centrifugations. The bars indicate the cruzipain content as compared with the respective controls, taken as 100%. (b) *T. cruzi* cells were incubated with [14 C]glucose for 2 min in the presence of increasing concentrations of DNJ and labeled material insoluble in hot 10% trichloroacetic acid counted. For further details see Materials and Methods.

did not affect the cellular general metabolism in long term incubations but also in short term ones: as depicted in Fig. 5 b, incubation of *T. cruzi* cells with DNJ concentrations up to 23-fold higher than those of [14 C]glucose for 2 min did not affect incorporation of label into hot 10% trichloroacetic acid insoluble material (mainly amino acids in proteins). This last result also strongly suggested that the inhibitors entered *T. cruzi* cells by facilitated diffusion and not through an hexose transporter, the same as has been reported to occur for the mannose analog 1-deoxymannojirimycin in mammalian cells (27).

Nevertheless, the cruzipain total content of lysosomes was partially affected by the drug: as shown in Fig. 5 a, inset, only 64%–75% of the proteinase was found in cells harvested from the DNJ-containing medium. This diminution in cruzipain content reflected an inhibition of the synthesis of the proteinase: *T. cruzi* cells were pulsed for 15 min with [35 S]methionine and chased with the unlabeled amino acid for 300 min in the presence or absence of DNJ. Label in cruzipain was quantitated in both the lumen of the endoplasmic reticulum and in lysosomes after the analytical procedure employed in Fig. 4 c. Results similar to those shown in that figure were obtained: whereas in the pulse samples of cells incubated with or without DNJ cruzipain appeared in the second supernatant (lumen of the endoplasmic reticulum), in the chase ones the proteinase mainly appeared in the first supernatants (lysosomes). Label in cruzipain in both the pulse and chase samples isolated from cells incubated with DNJ was \sim 30% lower than that obtained from cells incubated without the drug.

DNJ Causes a Delay in the Arrival of Cruzipain to Lysosomes

T. cruzi cells were pulse chased with [35 S]methionine in the presence or absence of DNJ, submitted to freezing and thawing and the supernatants of 15,000 g for 10-min centrifugations applied to Con A-Sepharose columns as de-

scribed above for the experiment shown in Fig. 4 c. Material eluting with α -methylmannoside was run on polyacrylamide gel electrophoresis under denaturing conditions. As shown in Fig. 6 a, cruzipain always appeared as a double band thus indicating that the lysosomal form of the enzyme was being analyzed. Label in the sample devoid of DNJ reached a plateau after \sim 100 min of chase, whereas that containing the drug did not reach it even after 300 min (Fig. 6 a). Quantitation of label in the bands confirmed this conclusion (Fig. 6 b).

Discussion

We have previously established that *T. cruzi* cells have a glucosidase II activity with characteristics similar to those of the mammalian enzyme, as (a) both had the same neutral optimum pH value, (b) both were more active on Glc₁Man₉GlcNAc than on Glc₁Man₄GlcNAc, and (c) similar amounts of the glucose analogs DNJ and castanospermine (5 μ M and 8 μ M, respectively) were required for attaining a 50% inhibition of both enzymes in cell free assays (3, 13).

Parasite cells grown in the presence of [14 C]glucose and 6 mM DNJ, or 2.6 mM castanospermine or of both inhibitors at the same concentrations appeared to have a single glucose unit in \sim 52–53% of whole cell N-linked oligosaccharides (13). The fact that not all the oligosaccharides appeared glucosylated could not be ascribed to the action of unspecific glucosidases not inhibited by the above mentioned compounds and slowly acting during storage of glycoproteins in their final destinations as the same percentage of glucosylated oligosaccharides was obtained in glycoproteins isolated from cells grown in the presence of the drugs or in those incubated with them and [14 C]glucose for only 60 min (13) (the doubling time for *T. cruzi* epimastigote cells is \sim 36 h). Moreover, a crude microsomal *T. cruzi* fraction did not degrade Glc₁Man₉GlcNAc in the

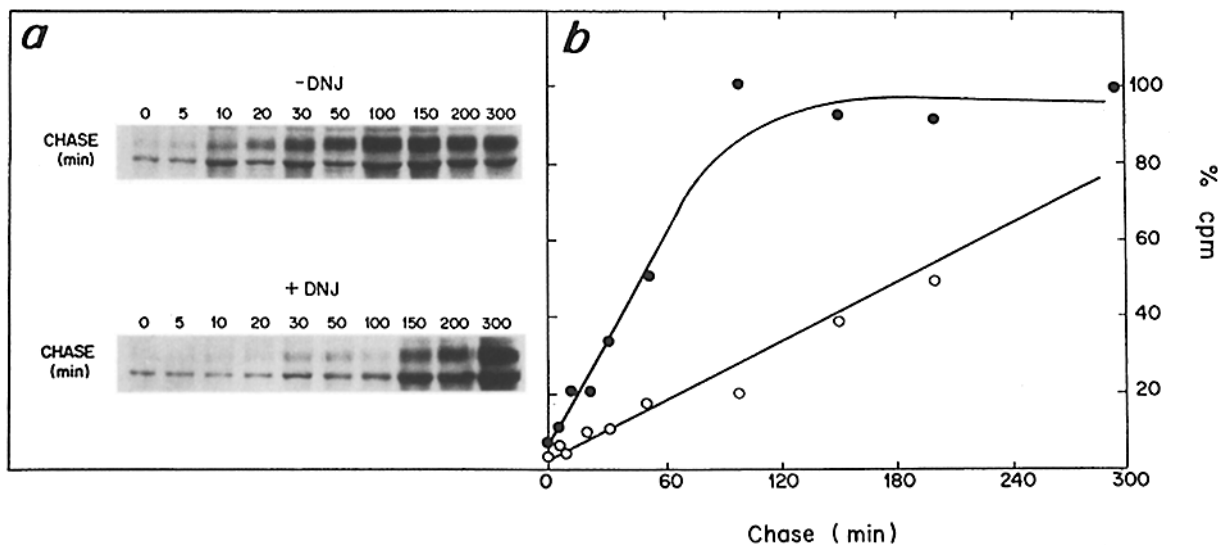


Figure 6. The effect of DNJ on the arrival of cruzipain to lysosomes. *T. cruzi* cells were incubated with [³⁵S]methionine in the absence (6 a, upper row) or presence (6 a, lower row) of 6 mM DNJ for 15 min after which they were centrifuged and incubated with the unlabeled amino acid. Aliquots were withdrawn after indicated chase periods, centrifuged, and submitted to freezing and thawing. The supernatants of 15,000 g for 10-min centrifugations were applied to Con A-Sepharose columns and material eluting with α -methylmannoside was run on 10% polyacrylamide gel electrophoresis under denaturing conditions. (b) Portions of gels in a containing cruzipain were excised and counted: \circ -- \circ , with DNJ and \bullet -- \bullet without the drug. Values obtained after 300 min chase (9,260 cpm without DNJ and 5,743 cpm with DNJ) were arbitrarily taken as 100%.

presence of the inhibitors, thus strongly suggesting that all activities degrading the substrate were sensitive to the drugs (13). In addition, *T. cruzi* cells were shown not to have the endomannosidase that liberates GlcMan from Glc₁Man₇₋₉GlcNAc₂ (13). This enzyme is not inhibited by castanospermine or DNJ and has been described to occur in the Golgi apparatus of certain mammalian cells (26). It may be concluded, therefore, that the percentage of glucosylated oligosaccharides found actually reflects the percentage of oligosaccharides that are glucosylated in vivo.

The oligosaccharides present at the single N-glycosylation site of the COOH-terminal domain of cruzipain were shown here to be glucosylated in some enzyme molecules and not in others. Therefore, the fact that only 52–53% of whole cell N-linked oligosaccharides were glucosylated was not apparently due to glucosylation being restricted to oligosaccharides linked to particular asparagine residues but to the fact that oligosaccharides linked to the same asparagine residue in a glycoprotein may be glucosylated in some glycoprotein molecules and not in others. This result is consistent with the known glucosylation requirement of the UDP-Glc:glycoprotein glucosyl-transferase for not properly folded protein moieties and the fact that folding of proteins in the endoplasmic reticulum is an asynchronous process. It is known that time required for attaining the final tertiary structure may differ among molecules of the same species and may be related to which chaperones the individual glycoproteins bind during folding (10). It may be assumed that rapidly folding cruzipain molecules would be glucosylated to lower extents than slower ones.

DNJ did not affect *T. cruzi* cell growth rate or the total content of soluble proteins. These results demonstrate that the inhibitor did not interfere with the parasite general metabolism in long term incubations. The same conclusion

could be drawn from short term ones as no effect on the incorporation of label into 10% trichloroacetic acid insoluble material (mainly amino acids in proteins) was observed when [¹⁴C]glucose was added for 2 min to *T. cruzi* cultures containing up to 23-fold higher concentrations of DNJ than those of the added monosaccharide.

Notwithstanding the lack of effect of DNJ on cell growth rate and on the parasite general metabolism, the inhibitor produced a 30% inhibition of cruzipain synthesis, a diminution in the same percentage in the lysosomal content of the enzyme and at least a threefold increase in the time required for arrival of half cruzipain molecules to lysosomes. As mentioned above, it is assumed that this delay reflects an increased permanence of cruzipain in the endoplasmic reticulum of inhibitor-containing cells. Inhibition of the synthesis of certain glycoproteins by DNJ has been observed before in mammalian cells (14, 22). The fact that synthesis and lysosomal content of cruzipain were diminished to the same extent strongly suggests that components essential for folding, processing, and transport of the lysosomal enzyme precursor were not significantly affected in DNJ-treated cells and that the much more significant increase in the time required to reach lysosomes was unrelated to the inhibition of synthesis.

It is worth mentioning that it has been reported several years ago that addition of DNJ to mammalian cells produced a delay in the secretion of glycoproteins or in their arrival to lysosomes (14, 22, 25). On the other hand, secretion of proteins as albumin was not affected by the drug, this result being consistent with the lack of effect on cellular general metabolism observed in this report (25). The delay in glycoprotein transport cannot be ascribed in mammalian cells to an interaction of calnexin with glucose-containing glycoproteins because, as mentioned above, in

mammalian and yeast cells glucosidase inhibitors produce an accumulation of protein-linked oligosaccharides containing two or three glucose residues that are not recognized by calnexin (16, 17, 20). The delay in glycoprotein transport observed was probably due to a deficient glycosylation of proteins: DNJ inhibits the formation of glucosylated dolichol-P-P derivatives and produces an accumulation of lipid-linked Man₉GlcNAc₂ (34). Mammalian and fungal oligosaccharyltransferases require the presence of glucose units in the oligosaccharide in order to catalyze an efficient transfer reaction (21). In fact, in one of the reports mentioned above it was found that lysosomal enzymes arriving to lysosomes in DNJ-treated cells had less oligosaccharide chains than under normal conditions (22). The inhibitory effect of DNJ on protein N-glycosylation was confirmed in other reports (14, 15). Folding of glycoproteins in the endoplasmic reticulum is heavily dependent on the presence of oligosaccharides (18). On the other hand, several *Saccharomyces cerevisiae* mutants that are unable to synthesize glucosylated dolichol-P-P derivatives have been described (1, 35). The mutants accumulated and transferred to proteins Man₉GlcNAc₂, the same as *T. cruzi*. Those yeast cells cannot be used for studies similar to those described here, however, as glycoproteins formed in them were found to be, as expected, heavily underglycosylated (1). Moreover, *S. cerevisiae* are the only cells described so far to be apparently devoid of the UDP-Glc:glycoprotein glucosyltransferase (12).

Drawbacks encountered with mammalian and yeast cells were obviated in the present study because: (a) no glucosylated dolichol-P-P derivatives are formed in this parasite (29), (b) the *T. cruzi* oligosaccharyltransferase does not require the presence of glucose residues in the oligosaccharide in order to catalyze an efficient transfer reaction (3), and (c) protein-linked oligosaccharides formed in *T. cruzi* in the presence of DNJ only contain a single glucose residue (13).

The results reported here are compatible with the model proposed for quality control of glycoprotein folding in the endoplasmic reticulum. It should be stressed, however, that not only an interaction of calnexin with cruzipain but also the existence in trypanosomatids of calnexin or other lectin-like proteins that recognize monoglucosylated oligosaccharides have not been demonstrated. Results presented only show that retention of the single glucose residue added by the UDP-Glc:glycoprotein glucosyltransferase to high mannose-type oligosaccharides causes a delay in the exit of glycoproteins from the endoplasmic reticulum. If such lectin-like molecules occur in trypanosomatids, their interaction with the monoglucosylated oligosaccharides should be loose enough to allow glucosidase II to remove the glucose unit and to allow passage of properly folded but still glucosylated glycoproteins (as in DNJ-treated cells) to the Golgi apparatus.

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