Isolation and characterization of the Golgi complex of the protozoan *Trypanosoma cruzi*

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SUMMARY

In this study the Golgi complex of the epimastigote forms of *Trypanosoma cruzi* were isolated and characterized. Using well-controlled sonication to rupture the cells and centrifugation on a discontinuous sucrose density gradient, a highly enriched Golgi fraction was obtained. The Golgi fraction contained most of the β-galactosyltransferase (β-Gal transferase) and UDP-*N*-acetyl-glucosamine: polypeptide-α-*N*-acetyl-glucosaminyltransferase (*O*-α-GlcNAc transferase) activities with minimal contamination of other organelles, as observed by enzymatic assays and electron microscopy analysis. To characterize the Golgi from *T. cruzi* cells further, it was incubated with a monoclonal antibody against a 58 kDa protein involved in the association of the Golgi complex with microtubules in mammalian cells. Immunofluorescence microscopy showed that the 58 kDa protein is localized in the *T. cruzi* Golgi region, a result confirmed by high resolution scanning electron microscopy immunocytochemistry. Thus, our results show, for the first time, that the β-Gal transferase, the *O*-α-GlcNAc transferase and the 58 kDa protein are present in the Golgi complex of *T. cruzi* and are novel biochemical markers which can be used in the characterization of this organelle in *T. cruzi*.

Key words: Golgi complex, subcellular fractionation, glycosyltransferases, *Trypanosoma cruzi*.

INTRODUCTION

One of the most important functions of the Golgi complex in higher organisms is related to its presence as a central structure within the cell through which newly synthesized secretory and membrane proteins are transported, modified, and sorted *en route* to their final destination inside or outside the cells (Donaldson *et al*. 1990). The striking morphology of this organelle, consisting of ordered stacks of cisternae, reflects a spatially differentiated structure that is believed to correlate with distinct Golgi functions (Palade, 1975; Farquhar, 1985; Dumphy & Rothman, 1985). Furthermore, several additional lines of evidence indicate that the Golgi complex is conserved throughout eukaryotes (Mollenhauer & Morré, 1996; Becker & Melkonian, 1996), although its cisternae are not so well organized in *Entamoeba* (Mazzuco, Benchimol & De Souza, 1997) and in *Giardia* (Luján *et al*. 1995). It is important to note that the vast body of information on the biochemical composition and physiology of this organelle was only accumulated after its isolation.

Despite the large amount of knowledge on pathogenic protozoa, particularly *T. cruzi* (De Souza, 1984, 1989), there are few data on their cell organelles. For instance, the reservosomes and the Golgi complex have only been characterized at the ultrastructural level. In the case of the Golgi complex in this parasite, it has been reported that its organization varies according to its multiple activities during the trypanosomal differentiation process, suggesting a dynamic structure (Figueiredo & Soares, 1995).

Biochemical studies have shown that in members of the Trypanosomatidae family the pathway of *N*-linked oligosaccharide processing for newly made proteins is similar to that found in other eukaryotic cells (Parodi, Quesada-Allue & Cazzulo, 1981; Parodi, Martin-Barrients & Engel, 1989; Parodi, 1993). This observation suggests that in trypanosomatids, the Golgi complex plays an analogous function in glycoprotein biosynthesis. Recently, a novel series of unique *O*-linked-*N*-acetylglucosamine (GlcNAc)-containing oligosaccharides have been characterized in sialoglycoproteins (mucin-like

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molecules) of *T. cruzi* (Previato *et al*. 1994, 1995; Almeida *et al*. 1994; Serrano *et al*. 1995; Todeschini *et al*. 2000). The biosynthesis of these *O*-GlcNAc-linked oligosaccharides is initiated by the transfer of α-GlcNAc unit to threonine residue on the peptide acceptor by an *O*-α-GlcNAc transferase (Previato *et al*. 1998). Although this enzyme was detected in crude microsomal membrane preparations from epimastigote and trypomastigote forms, its subcellular localization has not been defined.

Despite extensive information on the distribution, structure and biosynthesis of surface glycoconjugates in trypanosomatids (Ferguson, 1999), there are few reports of the isolation of homogeneous Golgi preparations. One study has reported a highly enriched Golgi fraction obtained from 3 species of African trypanosomes (Grab *et al*. 1984). This fraction, however, consisted predominantly of smooth surface vesicles and flattened cisternae, rather than stacked Golgi cisternae. In view of the important role that the Golgi complex is expected to play in the cell cycle and the pathways of glycoconjugate biosynthesis in *T. cruzi*, the isolation and biochemical characterization of this organelle is needed.

Here, we describe the isolation and characterization of a highly enriched Golgi complex fraction from axenically cultured epimastigotes of *T. cruzi*. We show that 2 associated glycosyltransferase activities and a protein antigenically cross-reactive with a mammalian 58 kDa protein are located in the Golgi complex of this parasite.

MATERIALS AND METHODS

Parasite

Epimastigote forms of the Y strain of *T. cruzi* were cultivated for 5 days at 28 °C in LIT medium (Camargo, 1964) supplemented with 10% heat-inactivated fetal calf serum.

Subcellular fractionation

All procedures were performed at 4 °C unless otherwise indicated. *T. cruzi* cells were harvested by centrifugation in a Sorvall RCB ultracentrifuge (GSA Rotor) at 1000 ***g*** for 10 min and washed 3 times in 10 mM Tris–HCl buffer, pH 7·4, containing 0·25 M sucrose and 2 mM $MgCl_2$ (G buffer). Approximately 10^{11} washed cells were resuspended in a hypotonic solution (G buffer without sucrose) containing a cocktail of protease inhibitors (1 mM PMSF, antipain 1 μg/ml, pepstatin A 1 μg/ml and 15 μg/ml benzamidine) and disrupted by sonication on ice with 15 cycles of 2 s with 1 s rest between cycles in an ultrasonic apparatus (Sigma, GEX 600 Model) using a standard probe (13 mm radiating diameter). This fraction was termed the homogenate fraction. The cell disruption was monitored by phase-contrast microscopy. A concentrated sucrose solution, prepared in 10 mM Tris–HCl buffer, pH 7·4, was immediately added to the homogenate fraction to a final concentration of 0·25 M to minimize osmotic damage. Lysed cells were centrifuged at 2500 ***g*** for 15 min and the pellet containing unbroken cells, nuclei, and kinetoplasts was discarded. The resultant post-nuclear supernatant (PNS) was harvested and an equal volume of 2·3 M sucrose added to give a final sucrose concentration of 1·4 M. This suspension (15 ml) was loaded at the bottom of ultracentrifuge tubes (Beckman SW 28) and overlaid in succession with 7 ml of 1·2 M sucrose, 7 ml of 1·0 M sucrose, and 7 ml of 0·8 M sucrose. All sucrose solutions were prepared in 10 mM Tris–HCl buffer, pH 7·4. This gradient was centrifuged in a L8M ultracentrifuge at 95000 ***g*** for 1 h 30 min using a Beckman SW 28 rotor (Palo Alto, CA, USA). The pellet and the bands formed were carefully removed with a Pasteur pipette and diluted 10 times with G buffer without sucrose, collected by centrifugation at 80000 ***g*** for 45 min (Beckman Type 65 rotor), and used for subsequent analysis.

Electron microscopy

For conventional electron microscopy analysis, aliquots of whole cells and subcellular fractions were fixed for 2 h at room temperature by the addition of an equal volume of 5% glutaraldehyde in 0·1 M cacodylate buffer, pH 7·4/0·25 M sucrose. Post-fixation was carried out in 1% osmium tetroxide in cacodylate buffer, containing 0·8% potassium ferricyanide and 5 mM $CaCl_2$, dehydrated in acetone, and samples were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed in a Zeiss CEM-900 electron microscope.

Immunofluorescence microscopy

Parasites fixed in freshly prepared 4% paraformaldehyde in 0·1 M cacodylate buffer, pH 7·2, were washed with PBS, and allowed to adhere for 30 min to coverslips previously coated with 0·1% poly-L-lysine. They were then permeabilized for 15 min with a solution of 0·3% Triton X-100, blocked 10 min with 50 mM NH_4Cl, and in 3% bovine serum albumin in PBS for 1 h, and finally incubated for 1 h with a mouse monoclonal antibody against the Golgi 58 kDa protein (clone 58k9, Sigma) (1 : 50 dilution). Following incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Sigma) (1 : 100 dilution), the coverslips were mounted on glass slides and immunofluorescence images obtained with a Zeiss Axioplan fluorescence microscope. Control preparations were incubated without the primary antibody.

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High resolution scanning electron microscopy

For immunoelectron cytochemistry, a sample of the Golgi fraction (GF) was fixed and processed for scanning electron microscopy. Briefly, after fixation, small drops of the sample were placed on a specimen support, incubated for 1 h in the presence of the monoclonal antibody recognizing Golgi 58 kDa protein (Sigma) (dilution 1 : 50) and for 30 min in the presence of rabbit anti-mouse IgG (1 :100) (BBInternational) labelled with 10 nm colloidal gold particles. Post-fixation was carried out in 3 steps: (i) for 30 min at 28 °C with 1 % osmium tetroxide in cacodylate buffer containing 0·8 % potassium ferricyanide and 5 mM $CaCl_2$; (ii) for 30 min at 28 °C with 1 % tannic acid in cacodylate buffer; (iii) for 30 min at 28 °C with 1 % osmium tetroxide in cacodylate buffer containing 0·8 % potassium ferricyanide and 5 mM $CaCl_2$. Subsequently, the samples were dehydrated in graded ethanol, critical point-dried in CO_2, coated with chromium in a Penning sputter system in a high vacuum chamber (Gatan-model 681), and viewed in a JEOL-JSM-6340F Field Emission Scanning Electron Microscope. Control preparations were incubated with the secondary antibody only. Images were obtained using secondary and backscattered electrons.

Electrophoretic analysis and protein blotting

The proteins of different subcellular fractions were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) in 10 % polyacrylamide (Laemmli, 1970), and electro-transferred to nitrocellulose sheets (Towbin Staehelin & Gordon, 1979), using a semidry transfer cell at 10 V (Bio-Rad, Richmond, CA, USA). Following transfer, the nitrocellulose was blocked in 0·5 % fish gelatin and 0·0 1 % Tween-20 in TBS for 4 h at room temperature. A dilution of 1 : 5000 of monoclonal mouse anti-58 kDa protein prepared in blocking buffer was then applied at room temperature for 60 min. The nitrocellulose was washed 3 times for 10 min each in TBS and incubated with the secondary antibody, alkaline phosphatase-conjugated rabbit anti-mouse IgG (Sigma) (1 : 5000 dilution). Immunoblots were visualized using the BCIP/NBT solution kit (Sigma) according to the manufacturer's instructions.

Preparation of acceptor substrate for T. cruzi *β-Gal transferase*

GlcNAc-rich peptide obtained from purified sialoglycoproteins of *T. cruzi* by Smith degradation (Previato *et al*. 1998) was used as acceptor substrate for *β*-Gal transferase. Briefly, to 2 ml of a solution containing 30 mg of sialoglycoproteins in 0·1 M sodium acetate, pH 4·6, was added an equal volume of 0·2 M $NaIO_4$. After 24 h at 4 °C, the reaction was interrupted by addition of glycerol. The oxidized product was recovered by gel filtration chromatography on a column of Sephadex G25 SF (1 × 10 cm Pharmacia) eluted with water at a flow rate of 1 ml/min, and reduced with sodium borohydride for 3 h at room temperature. Boric acid was removed by repeated addition of methanol and evaporation to dryness. The oxidized and reduced material was submitted to partial acid hydrolysis with 0·02 M trifluoracetic acid for 30 min at 100 °C. The resulting GlcNAc-rich peptides were recovered by gel filtration chromatography on Sephadex G25 SF column, as above.

Enzymatic assays

In most cases, the fractions were assayed immediately, occasionally they were frozen at −70 °C until use. All assay conditions were optimized with regard to different kinetic parameters (e.g. substrate and protein concentrations, and pH). In order to obtain complete lysis of organelles the assays were performed in the presence of 0·1 % Triton X-100.

Hexokinase. This marker enzyme of glycosomes was measured according to Joshi & Jagannathan (1968). The reaction mixture consisted of 10 mM glucose, 0·6 mM NADP, 10 mM $MgCl_2$, 0·1 mM Tris–HCl, pH 7·4, 0·64 mM ATP, 10 μg/ml glucose-6-phosphate dehydrogenase and water in a total volume of 1·0 ml. The reaction was initiated with addition of 30 μl of sample and monitored spectrophotometrically at 340 nm.

Acid phosphatase. The method described by Tosomba, Coetzer & Londsdale-Eccles, (1996) was used. The reaction mixture consisted of 20 μl of 100 mM sodium acetate buffer, pH 5·0; 5 μl of 50 mM *p*-nitrophenol (*p*-NPP) solution made up in 100 mM sodium acetate buffer, pH 5·0, and 10–20 μg of enzyme protein solution in a total volume of 50 μl. Incubations were carried out for 60 min at 37 °C with gentle shaking. The reaction was stopped by the addition of 200 μl of 10 mM NaOH. The amount of *p*-NPP liberated was determined spectrophotometrically at 405 nm.

O-α-GlcNAc transferase. The *O*-α-GlcNAc transferase involved in the addition of α-GlcNAc to threonine residues in the sialoglycoproteins of *T. cruzi* was measured according to the method of Previato *et al*. (1998). The reaction mixture contained 25 mM Tris–HCl buffer (pH 7·4), 5 mM $MnCl_2$, 0·1 % Triton X-100, 1·5 μCi of UDP-[^{3}H]GlcNAc (40–60 Ci/mmol), and 6·8 nmol synthetic peptide acceptor substrate [$KP_2T_8KP_2$] in a final volume of 50 μl. The reaction was initiated by addition of enzyme sources (250 μg protein of whole homogenate; 190 μg protein of post-nuclear supernatant and 35 μg of Golgi fraction). The mixture was

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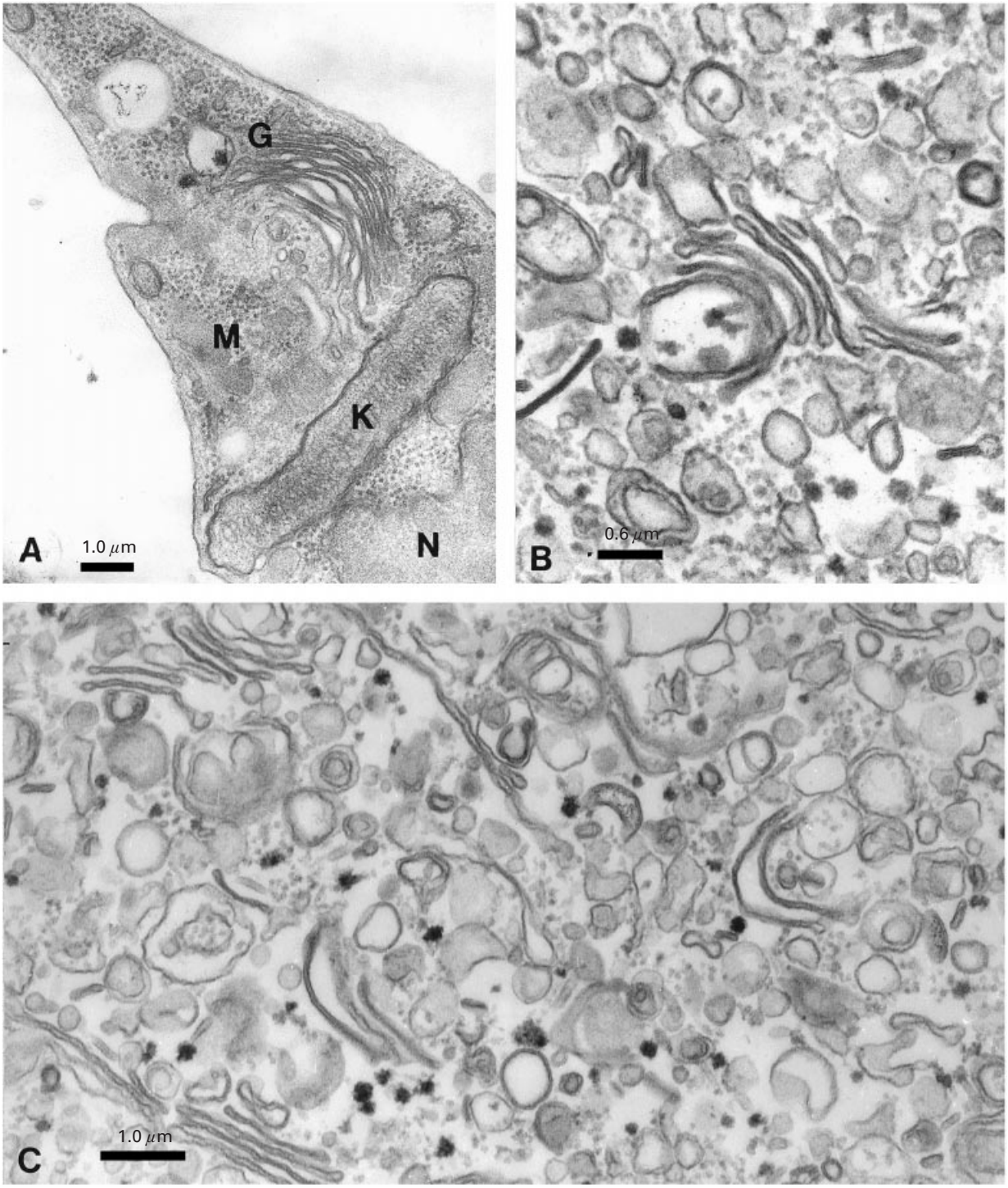

Fig. 1. Electron microscopy of subcellular fractions. (A) Electron micrograph showing the anterior region of an epimastigote form of *Trypanosoma cruzi*. G, Golgi apparatus; K, kinetoplast; N, nucleus, M, mitochondria. (B) High magnification image of the post-nuclear supernatant fraction showing a Golgi complex with several stacked cisternae. (C) General view of the Golgi fraction (GF) showing Golgi complex cisternae and several Golgi cisternae profiles.

incubated at 28 °C for 30 min, and reaction interrupted by addition of 950 μl of 50 mM formic acid. The reaction mixture was loaded onto a sulfopropyl-Sephadex column (SP-C 25–120, Pharmacia Biotech) equilibrated in 50 mM formic acid. The column was washed with 50 mM formic acid and the peptide and labelled glycopeptide were eluted with 0·5 M NaCl. The incorporation of [^{3}H]GlcNAc into the peptide was determined on aliquots of the eluate by liquid scintillation counting after addition of Bray solution. Control assays without the acceptor peptide were performed to correct for endogenous activity.

β-Gal transferase. The *β-Gal* transferase activity was determined in reaction mixtures containing 25 mM, Tris–HCl, pH 7·4, 5 mM $MnCl_2$, 0·1 %

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Table 1. Enzyme activities in subcellular fractions of *Trypanosoma cruzi*

(A typical experiment is shown here in which each number represents the mean of duplicate determinations. The experiments were repeated at least 3 times with minor modifications in the results.)

Assay	Homogenate fraction	PNS	GF
Enzyme	Activity*†		
NADPH cyt c reductase*	0·25	0·34	0·043
Acid phosphatase*	3·0	4·5	15·2
5′-nucleotidase*	0·101	0·157	0·06
Succinate cyt c reductase*	0·07	0·04	0·03
Hexokinase*	0·032	0·0436	0·0034
O-α-GlcNAc transferase†	0·076	0·248	2·044
Gal transferase†	1·28	6·56	114·80

*Specific activity expressed as micromol of product released per min per milligram of protein.
†Specific activity expressed as picomol of [^{3}H]GlcNAc or [^{3}H]Gal transferred per h per milligram of protein.

Triton X-100, 10 μCi of [^{3}H]UDP-Gal (40–60 Ci/mmol), and 200 μg of *T. cruzi*-derived GlcNAc-rich glycopeptide (acceptor substrate) in a final volume of 50 μl. The reaction was initiated by addition of the enzyme sources (360 μg protein of whole homogenate; 135 μg protein of post-nuclear supernatant and 36 μg of Golgi fraction), and the mixture incubated at 28 °C for 30 min. The reaction was interrupted by addition of 950 μl of 50 mM formic acid, insoluble material was removed by centrifugation for 5 min in an Eppendorf microcentrifuge, and the supernatant loaded onto a 1 ml sulfopropyl-Sephadex column (SP-C50, Pharmacia Biotech) equilibrated in 50 mM formic acid. The column was washed with 10 ml of 50 mM formic acid and the GlcNAc-rich peptide and the labelled GlcNAc-rich peptide were eluted with 5 ml of 0·5 M of NaCl. Incorporation of [^{3}H]Gal into the GlcNAc-rich peptide was determined on aliquots of the eluate by liquid scintillation counting after addition of Bray solution (10 ml). Control assays without the acceptor substrate were performed to correct for endogenous activity.

Characterization of in vitro *galactosylated GlcNAc-rich peptide*

Incorporation of [^{3}H]Gal into GlcNAc-rich glycopeptides recovered from the SP-Sephadex column was confirmed by β-elimination (Yen & Ballou, 1974) and acid hydrolysis. Salt-free labelled [^{3}H]Gal–GlcNAc-rich glycopeptides were dissolved in 0·01 M NaOH containing 0·3 M $NaBH_4$ and incubated at 37 °C for 48 h to β-eliminate *O*-linked glycans. The solution was neutralized with 2 M acetic acid and passed through Dowex 50W-X8 (25–50 mesh H^+ form). Boric acid was removed by repeated additions of methanol and evaporation to dryness. The residue was dissolved in distilled water and analysed by gel filtration chromatography on a BioGel P-4 column (extra fine, Bio-Rad, 120 cm × 0·5 cm) with 0·2 M ammonium acetate as eluent. The column was calibrated using bovine serum albumin, and [^{14}C(U)](Glc) (2–10 mCi/mmol) as markers for the void and included volumes, respectively, and with authentic standards of Galpβ1–4[^{3}H]GlcNAcO and Galpβ1–6(Galpβ1–4)[^{3}H]-GlcNAcO as markers for monosaccharide- and disaccharide-HexNAcO (Previato *et al*. 1998). Fractions of 300 μl were collected and assayed for radioactivity. The labelled peak migrating as monosaccharide-*N*-HexNAcO was pooled, desalted by passage through a BioGel P-2 column (extra fine, BioRad, 50 cm × 0·8 cm) and lyophilized. The desalted labelled monosaccharide-HexNAcO was further analysed by RP-HPLC on a porous graphitized carbon (PGC) column (Hypercarb, 7 μm, 200 × 4·6 mm; Life Sciences International, Basingstoke, UK), calibrated with authentic Galpβ-1–4[^{3}H]GlcNAcO and Galpβ1–3[^{3}H]GlcNAcO and eluted with an aqueous acetonitrile gradient. Solvent A was 5 % acetonitrile in water and Solvent B 100 % acetonitrile. The linear gradient was started 5 min after injection from 0 to 15 % solvent B over 15 min (held until 20 min), and 0·5 ml fractions were collected. The flow rate was 0·5 ml/min. Aliquots of 50 μl were mixed with 5 ml of Bray solution and the radioactivity was determined by liquid scintillation counting. The RP-HPLC (PGC) purified labelled monosaccharide-HexNAcO fraction was hydrolysed with 2 M TFA 3 h at 100 °C. The TFA was evaporated *in vacuo*, and the radio-isotope labelled product analysed by Dionex HPLC using a PA-10 4 mm (10–32) column. The column was calibrated with authentic standards of [^{14}C(U)]galactose (Gal) (250–350 mCi/mmol), [^{14}C(U)]glucose (Glc) (2–10 mCi/mmol) and, [1-^{14}C]mannose (Man) (50–60 mCi/mmol) from American Radiolabeled Chemicals, Inc. The labelled monosaccharides were eluted with deionized water at a flow rate of 1·0 ml/min. Fractions of 0·5 ml were collected and 200 μl aliquots were mixed with 5·0 ml of Bray solution and counted for radioactivity.

Other procedures

5′-Nucleotidase, NADPH cytochrome c reductase and succinate cytochrome c reductase were assayed as previously described by Díaz, Monteiro-Leal & De Souza (1996) and Motta *et al*. (1997). Protein content was estimated using the Bio-Rad protein kit with bovine serum albumin a standard.

RESULTS

A general view of the anterior region of *T. cruzi* is shown in Fig. 1A. The protozoan possesses a single

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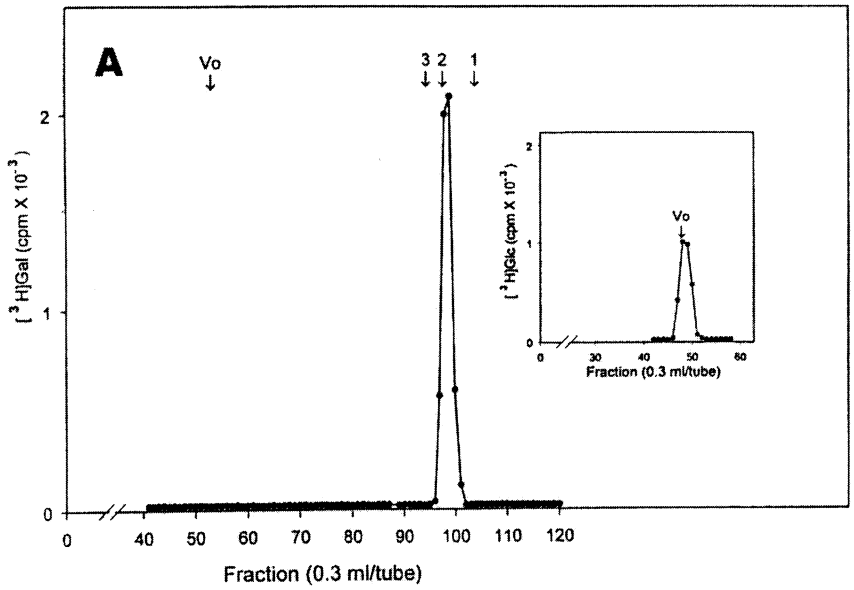

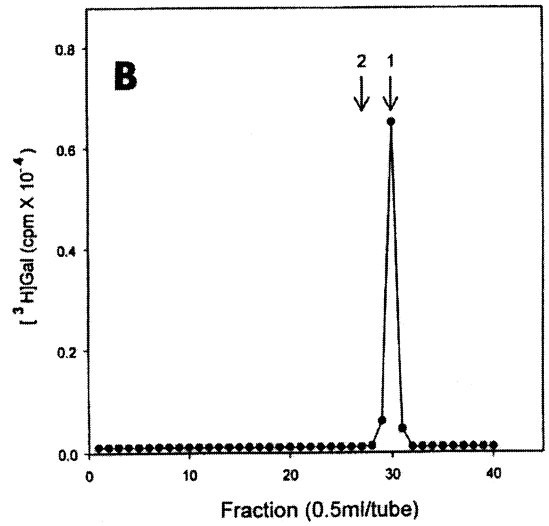

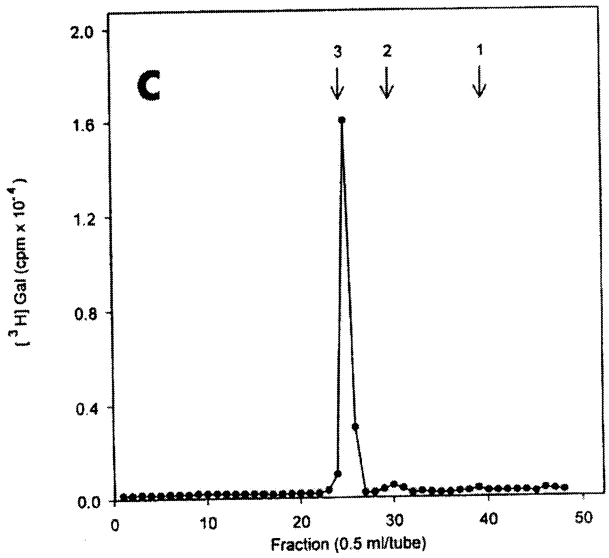

Fig. 2. Chromatographic profiles of reductive β-elimination product from *in vitro* incorporation of [^{3}H]Gal on GlcNAc-rich peptide by *Trypanosoma cruzi* β-Gal transferase. (A) Gel filtration on Bio-Gel P-4 column. The elution positions of authentic standards BSA (Vo), [^{3}H]Glc (1), Gal$p\beta$1-4[^{3}H]GlcNAcO (2), and Gal$p$1-6(Gal$p\beta$1-4)[^{3}]GlcNAcO (3) are indicated by arrows. The inset shows the elution position of intact [^{3}]Gal-GlcNAc-

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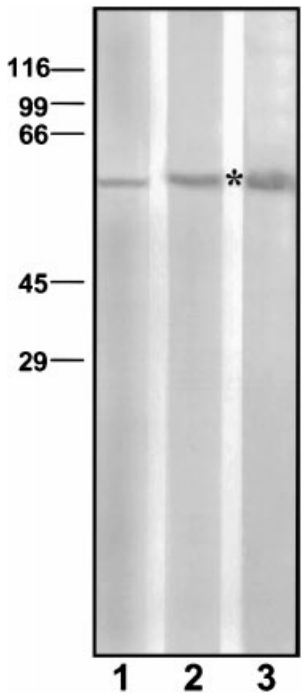

Fig. 3. Localization of the 58 kDa protein in different fractions obtained during the isolation of the Golgi complex of *Trypanosoma cruzi*. The fractions were subject to SDS–PAGE (10% gel), transferred to nitrocellulose membranes and probed with a monoclonal antibody that recognizes the Golgi 58 kDa protein. Homogenate fraction (lane 1), PNS (lane 2), GF (lane 3). Each lane contained 10 μg of total protein. Molecular weight markers are shown on the left.

Golgi complex formed by 4–10 stacked cisternae generally localized in the vicinity of the flagellar pocket. It is possible to observe several uncoated vesicles close to the most trans region of the Golgi complex.

Isolation and ultrastructural characterization of the highly enriched-Golgi complex fraction

The methodology used for isolation of the *T. cruzi* GF was based on the procedure of Díaz *et al*. (1996) to obtain the Golgi complex from *Tritrichomonas foetus*, with modified conditions for disruption of the cells. Using the procedure described here, 90% of cells were disrupted, as ascertained by phase-contrast microscopy where intact cells can be easily distinguished from the disrupted ones. Transmission electron microscopy analysis of the whole homogenate showed that the Golgi complex, and other structures such as mitochondria, nuclei, reservosomes and kinetoplasts, seemed to be well preserved with no morphological indications of membrane lesion (not shown). Although some nuclei were disrupted, releasing chromatin that spread in the field, this did not interfere with the fractionation yield. Fig. 1B, shows the PNS fraction in high magnification. A whole Golgi complex may be clearly seen released in this step of the fractionation procedure.

A band recovered by flotation at a position corresponding to the 1·0–1·2 M sucrose interfaces (named GF), after discontinuous sucrose gradient centrifugation, was enriched in Golgi complex elements, as determined by electron microscopy. Electron micrographs of the GF showed patterns typical of the Golgi complex with 3 or more opposed cisternae and often associated with vesicles (Fig. 1C). The cisternal contents were of moderate density. The other principal components of the fraction consisted of smooth-surface vesicles of various sizes containing little or no internal density. Some electron-dense granules were also observed, but organelles such as reservosomes, flagellae and rough endoplasmic reticulum were not observed.

Biochemical characterization of the Golgi fraction (GF)

The distribution of enzymes in the different fractions of *T. cruzi* epimastigotes obtained by discontinuous gradient sucrose centrifugation, is given in Table 1. Yields of Golgi-enriched fraction were typically 0·5–1·0 mg/l of cell culture. Acid phosphatase activity was found in considerably higher amounts (5-fold) in the GF compared with the homogenate fraction, although elevated activity was also observed in the PNS fraction. Contamination with other enzyme markers of cell compartments, such as NADPH cytochrome c reductase (endoplasmic reticulum), 5'-nucleotidase (plasma membrane), succinate cytochrome c reductase (mitochondria) and hexokinase (glycosome), was minimal in the GF fraction (0·13, 0·58, 0·42 and 0·10-fold, respectively), compared to that observed in the homogenate fraction. Table 1 also shows a distribution of glycosyltransferase activities (*O*-α-GlcNAc and β-Gal transferases) in the subcellular fractions obtained from *T. cruzi*. These enzymes, have been characterized to participate in the initial steps of *O*-linked glycosylation of *T. cruzi* sialoglycoproteins (Previato *et al*. 1998), were found to be 27- and 89-fold higher in GF when compared to the whole homogenate.

glycopeptide. The arrow indicates the void volume, using BSA as standard. (B) RP-HPLC on PGC column. Authentic standards of Gal$p$1-4[^{3}H]GlcNAcO (1) and Gal$p\beta$1-3[^{3}H]GlcNAcO (2) are indicated by arrows. (C) Dionex HPLC profile of [^{3}H]Gal released by acid hydrolysis of [^{3}H]Gal-GlcNAcO obtained after reductive β-elimination of [^{3}H]Gal-GlcNAc-glycopeptide. The standards of [^{14}C]Man (1), [^{14}C]Glc (2), and [^{14}C]Gal (3) are indicated by arrows.

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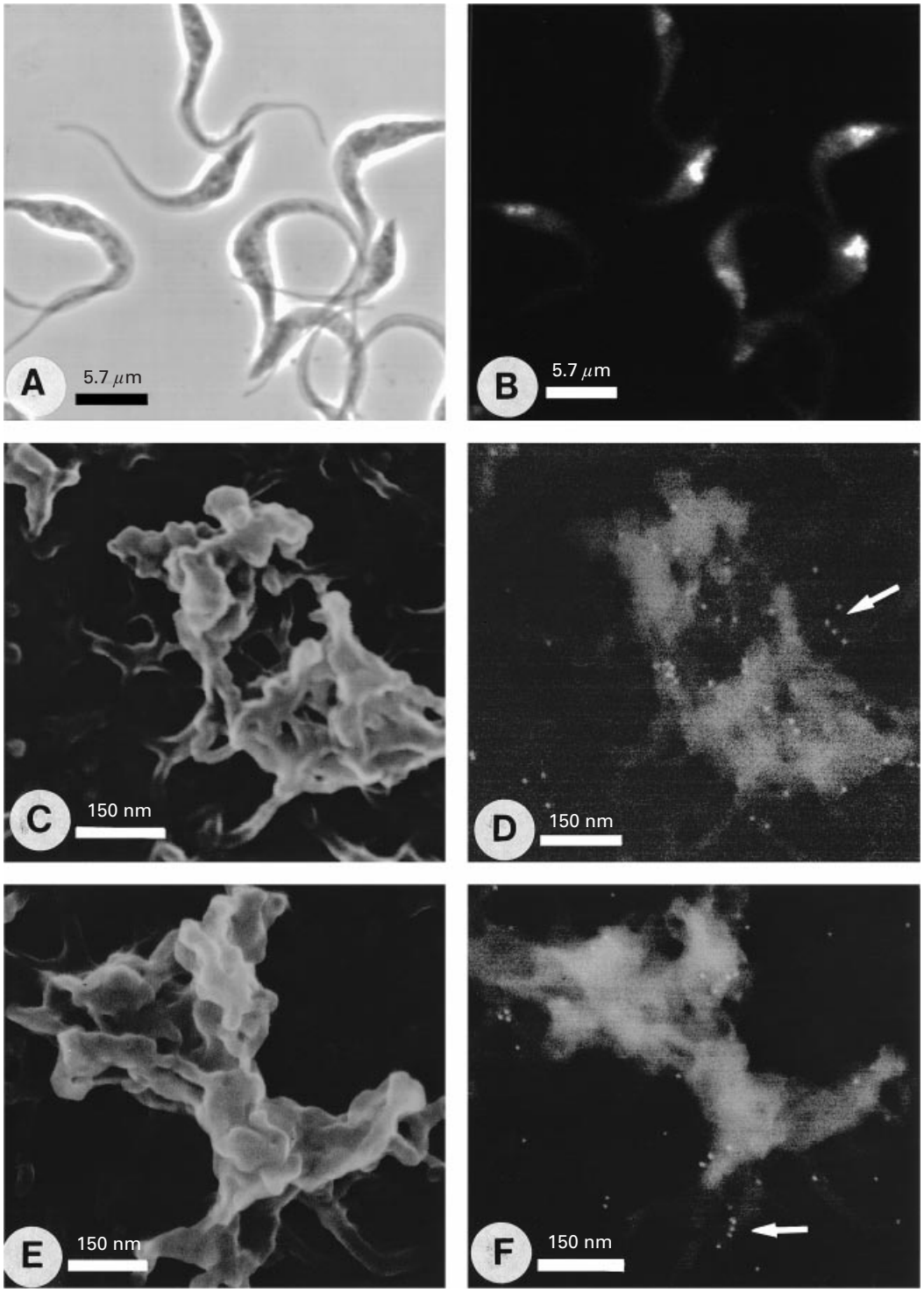

Fig. 4. Localization of 58 kDa protein in the Golgi complex of *Trypanosoma cruzi*. (A and B) Immunofluorescence microscopy showing perinuclear labeling of 58 kDa protein. (A) Shows the same cell as in (B) by bright field microscopy. (C and E) Golgi complex with several elements identified as smooth tubulo-vesicular structures, as seen by high resolution scanning electron microscopy of the GF fraction (secondary electron image). (D and F) Corresponding backscattered electron image of the fraction labelled with antibodies recognizing the 58 kDa protein, revealed using gold particles, which appear as light dots (arrows).

Isolation of β-eliminated product of galactosylated-GlcNAc-rich glycopeptide and characterization of labeled Gal

To confirm the incorporation of labelled Gal into the GlcNAc-rich peptide, the product of the β-Gal transferase assay was recovered by SP-Sephadex column chromatography and submitted to a reductive β-elimination reaction. The isolated radioactive glycan eluted in the same volume as authentic monosaccharide-HexNAcO on BioGel P-4 (Fig. 2 A). The identity of this labelled monosaccharide-

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alditol was determined by HPLC on the PGC column, on which it co-eluted with authentic Galpβ1 → 4[^{3}H]GlcNAcO, but separate from Galpβ1 → 3[^{3}H]GlcNAcO (Fig. 2B). To confirm that the radioactive residue incorporated into the GlcNAc-rich peptide was Gal, and had not undergone epimerization to Glc, the salt-free β-elimination product was acid hydrolysed and separated by Dionex-HPLC. All of the radioactivity comigrated with the Gal standard (Fig. 2C).

Localization of the 58 kDa protein at the Golgi complex

Several approaches were used to investigate the localization of the 58 kDa protein in *T. cruzi* using an antibody which recognizes a similar protein in mammalian cells. First, immunofluorescence microscopy of antibody-labelled whole cells showed that the labelling was restricted to the perinuclear region, where the Golgi complex is located (Fig. 3A and B). No fluorescence was observed when control parasites were incubated only with the secondary fluorescein-labelled goat anti-mouse-IgG (data not shown). Secondly, to analyse in more detail the structures labelled by the antibody, immunoelectron microscopy was performed on the GF fraction. Fig. 3C and E show images obtained by field emission high-resolution scanning electron microscopy, in which it is possible to visualize the 3-dimensional structure of the Golgi apparatus of the elements. These were identified as smooth tubule-vesicular structures forming a complex network. The smaller tubules radiated from a central organelle core towards to the edge. Backscattered electron imaging was used to display the heavy metal staining of these structures and allowed direct visualization of gold particles indicating localization of the 58 kDa protein present in the Golgi complex elements (Fig. 3D and F). Controls performed using only the secondary antibody showed no labelling in these structures (not shown). Thirdly, the antibody was also used to evaluate the 3 representative fractions obtained in the subcellular fractionation, using Western blot. The antibody to the 58 kDa protein recognized a major band of 58 kDa in all fractions, nevertheless higher immunoreactivity was observed in the GF (Fig. 4 lanes 1–3).

DISCUSSION

Previous morphological studies have shown that the Golgi complex of *T. cruzi* is a single structure formed by 4–10 stacked cisternae (Figueiredo & Soares, 1995). The isolation of the Golgi from *T. cruzi* is complicated by the difficulty of rupturing the parasite and the morphological characteristics of this organelle. It is important to develop a methodology that preserves Golgi with minimum damage during cell disruption. Our procedure, combining controlled sonication for cell rupture and purification by sucrose density gradient centrifugation, resulted in a Golgi complex preparation almost devoid of contamination by other organelles. Electron microscopy analysis of this fraction (GF) showed that it was highly enriched in elements of the Golgi complex. No contamination by flagellar, ribosomal or other organelles was observed, clearly suggesting that our methodology efficiently eliminated contaminating structures from the Golgi fraction.

The purity of GF was also evaluated by assaying β-Gal transferase activity, considered one of the most reliable Golgi markers (Farquhar & Palade, 1981). The data obtained on GF using this marker enzyme demonstrated that it has the highest activity, compared to the homogenate fraction. The expression of Gal-containing glycoproteins in *T. cruzi* epimastigote forms has been described (Previato *et al*. 1994, 1995). Recently, an *O*-α-GlcNAc transferase, involved in the *O*-glycosylation of mucin-like molecules of *T. cruzi* has been assayed (Previato *et al*. 1998). This enzyme catalyses addition of *O*-linked α-GlcNAc to threonine residues on the cell surface sialoglycoproteins of *T. cruzi*. An enrichment of 27-fold of *O*-α-GlcNAc transferase activity was found in the GF. Previously, the activity of this unique *O*-α-GlcNAc transferase was determined in microsomal membrane preparations from infective and non-infective forms of this parasite, suggesting that this enzyme was associated with some compartment of the secretory pathway. Our present results show that *O*-α-GlcNAc transferase is also a constituent of the Golgi complex, and hence a novel marker for this organelle in *T. cruzi*.

Of the other enzymes assayed, only acid phosphatase displayed increased activity (by 5-fold) in the GF fraction. This is in agreement with previous studies showing that this enzyme is localized in lysosome-like structures, the flagellar pocket, in cisternae and vesicles of the Golgi, as well as in Golgi-enriched fractions isolated from *T. brucei* (Langreth & Balber, 1975; Grab *et al*. 1984). Acid phosphatase activity has also been detected using cytochemical methods at the Golgi complex of other trypanosomatids (Pimenta & De Souza, 1986). In contrast, Figueiredo & Soares (1995) obtained inconsistent or negative results with epimastigote forms of *T. cruzi*. Taken together, our results indicate that a highly enriched Golgi complex fraction was obtained from the *T. cruzi* epimastigote forms.

Using 3 different approaches (immunofluorescence, immunoblotting and immunocytochemistry monitored by high resolution scanning electron microscopy), we were able to demonstrate that the GF was the primary immunoreactive site in *T. cruzi* for the antibody. Previous studies have indicated that the structural integrity and intracellular location of the Golgi complex in mammalian cells is de-

pendent upon the presence and organization of microtubules (Kupfer, Louvard & Singer, 1982; Wehland *et al*. 1983; Rogalski & Singer, 1984; Nemere, Kupfer & Singer, 1985; Tassin *et al*. 1985). It was later demonstrated that the behaviour of these distinct cytoplasmic structures is closely coordinated by a specific protein involved in linking the Golgi to microtubules (Bloom & Brashear, 1989). It has been suggested that the *in vivo* function of the 58 kDa protein is to provide an anchorage site for microtubules on the outer surface of the Golgi in mammalian cells (Bloom & Brashear, 1989).

In conclusion, our results demonstrate that the methodology here described is a relatively simple means to isolate the Golgi complex of *T. cruzi* as a source of material for structural and biochemical studies. The data presented in this paper also provide further insights into the pathway of protein glycosylation in this protozoan.

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