Identification of two distinct galactosyltransferase activities acting on the variant surface glycoprotein of *Trypanosoma brucei*

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Variant surface glycoproteins (VSGs) of *Trypanosoma brucei* contain two distinct glycosylation sites: (1) *N*-linked glycans within the protein portion of the molecules, and (2) the glycosyl-phosphatidylinositol (GPI) membrane anchor. Since galactose residues show uncommon α -glycosidic linkages in the GPI membrane anchor, we were prompted to investigate galactosylation of the GPI anchor. On comparing a trypanosome clone galactosylated exclusively in *N*-glycans (clone MITat 1.5) with clones galactosylated predominantly in the glypiated membrane anchor (clones MITat 1.4, MITat 1.6 and AnTat 1.8), clone MITat 1.5 showed a 10-fold increased enzyme activity when using a protocol including Triton X-100 to assay UDPgalactose:*N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase (EC 2.4.1.38). Only the VSG of clone MITat 1.5 could be radiochemically labelled with UDP[¹⁴C]galactose, and galactosylation of *N*-glycans was confirmed by digestion with peptide-*N*⁴-(*N*-acetylglucosaminyl)asparagine amidase (PNGase F). However, in a modified enzyme assay without detergent, galactosyltransferase activity was increased considerably (15-fold) in clone MITat 1.4. VSG galactosylation of clones MITat 1.4, MITat 1.6 and AnTat 1.8 was readily detected by fluorography of the respective SDS/polyacrylamide gels, suggesting that galactosyltransferase activity modifies the VSG membrane anchor in these clones. In this case, [¹⁴C]galactose labelling of immunoprecipitated VSG (clone MITat 1.4) was resistant to the release of *N*-glycans by PNGase F treatment, and thus revealed galactosylation *in vitro* of a VSG membrane anchor. Exoglycosidase digestions of VSG MITat 1.4 confirmed the presence of α -linked galactose residues. We suggest that these specific α -galactosyltransferases are inhibited by the action of detergent, but can be activated in a detergent-free buffer system.

INTRODUCTION

Variant surface glycoproteins (VSGs) of African trypanosomes, which form a protective barrier around each parasite and permit a sustained infection by means of antigenic variation (Borst, 1986), belong to a class of membrane proteins characterized by a common glycosyl-phosphatidylinositol (GPI) membrane anchor linked covalently to the C-terminus (Ferguson & Williams, 1988). All VSG molecules contain two distinct glycosylation sites: one or more *N*-linked internal glycans (Holder & Cross, 1981; Holder, 1985), and the glypiated membrane anchor (Cross, 1990).

VSGs can be divided into three subgroups (types I, II and III) on the basis of amino acid sequence similarities within the C-terminal third of the molecule (Holder & Cross, 1981; Holder, 1985). Whereas in type I VSGs (MITat 1.4 and MITat 1.6) internal glycans were found to be almost exclusively of the classical oligomannose type (Zamze *et al.*, 1990), type II (MITat 1.2) and type III (MITat 1.5) VSGs additionally contain bi-antennary complex-type oligosaccharides, and in type II VSGs a novel group of branched poly-*N*-acetyl-lactosamine oligosaccharides was recently shown (Zamze *et al.*, 1991). The two latter structures include galactose residues β 1,4-glycosidically linked to *N*-acetylglucosamine, and are partially substituted with α 1,3-linked galactose residues (Zamze *et al.*, 1991). Interestingly, complex-type oligosaccharides showed very rapid kinetics of endo- β -*N*-acetylglucosaminidase H resistance, suggesting an alternative glycosylation pathway in African trypanosomes (Bangs *et al.*, 1988). However, VSGs pass through the Golgi compartment on their way to the plasma membrane (Duszenko *et al.*, 1988), and it has been shown that UDPgalactose:*N*-acetylglucos-

aminyl glycopeptide β 1,4-galactosyltransferase activity is located within the Golgi apparatus in *Trypanosoma brucei* (Steiger *et al.*, 1980; Grab *et al.*, 1984), as it is in other eukaryotic cells (Kornfeld & Kornfeld, 1985).

The membrane anchors of *T. brucei* clones MITat 1.4 and MITat 1.6 have been sequenced (Ferguson *et al.*, 1988), showing that galactose forms Gal- α 1,2-Gal-, Gal- α 1,6-Gal- or Gal- α 1,3-Man linkages, thus leading to antennary structures. Interestingly, the observed α -glycosidic linkages have not been found in mammalian cells so far, and apparently depend on specific α -galactosyltransferases.

In the present study we have investigated the galactosyltransferases which modify *N*-glycans or the GPI anchor of VSG molecules in *T. brucei*, using trypanosome clones galactosylated either in internal glycans of their VSG molecules (such as clone MITat 1.5) or in their GPI anchor (such as clones MITat 1.4, MITat 1.6 and AnTat 1.8) (see Table 1). Although clone AnTat 1.8 is derived from a different trypanosome strain, it apparently expresses the same VSG antigen type as does clone MITat 1.4 (Cross, 1977; Matthyssens *et al.*, 1981). Clone MITat 1.2 has been used for comparison only, since its VSG molecule is galactosylated at both sites (Table 1).

Part of this work was presented at the Annual Meeting of the Deutsche Gesellschaft für Zellbiologie (Göttingen, 3–8 March 1991) (Pingel & Duszenko, 1991).

MATERIALS AND METHODS

Materials

UDP-D-[U-¹⁴C]galactose (150–330 Ci/mol) and [³⁵S]methionine (1150 Ci/mmol) were purchased from Amersham–Buchler

Abbreviations used: (s)VSG, (soluble) variant surface glycoprotein; GPI, glycosyl-phosphatidylinositol; BmGT, UDPgalactose:*N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase from bovine milk (EC 2.4.1.38); PNGase F, peptide-*N*⁴-(*N*-acetylglucosaminyl)asparagine amidase (EC 3.5.1.52).

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(Braunschweig, Germany). Bovine milk UDPgalactose:*N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase (BmGT; EC 2.4.1.38) was from Sigma Chemie (Deisenhofen, Germany), coffee bean α -galactosidase (EC 3.2.1.22), bovine testes β -galactosidase (EC 3.2.1.23) and peptide-*N*⁴-(*N*-acetylglucosaminyl)-asparagine amidase of *Flavobacterium meningosepticum* (PNGase F; EC 3.5.1.52) were from Boehringer (Mannheim, Germany). All other reagents used were of analytical grade.

Preparation of trypanosomes

Trypanosome variants of Molteno Institute Trypanozoon antigenic types 1.2, 1.4, 1.5 and 1.6 (otherwise referred to as clones 221, 117, 118 and 121; see Table 1) of *T. brucei* strain 427 and AnTat 1.8 (Antwerpen Trypanozoon antigenic type) were purified from infected rat blood as previously described (Cross, 1975). Procyclic forms of clone MITat 1.4 were transformed from bloodstream forms by the protocol of Hamm *et al.* (1990). Soluble-form VSG (sVSG) was prepared by the method of Cross (1984).

Galactosyltransferase assays

Assay A. To assay UDPgalactose:*N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase, the following system (slightly modified from that of Steiger *et al.*, 1980) was used. Cells [(1–3) $\times 10^8$] were lysed in 100 mM-Tris/HCl buffer (pH 7.0) containing 1.6% (v/v) Triton X-100, 0.15 mM-UDPGalactose, 0.05 μ Ci of UDP[¹⁴C]galactose, 10 mM-MnCl₂, 5 mM-MgCl₂, 10 mM-ATP, 1 mM-dithiothreitol, 0.02% (w/v) Na₂S₂O₃ and protease inhibitors (chymostatin, leupeptin and pepstatin; each at 1 μ M) in a total volume of 100 μ l. In experiments with exogenous acceptors, ovalbumin (1%, w/v) or MITat 1.5 sVSG (0.1%, w/v) was added to the assay. BmGT (1 munit) was used in the appropriate experiments. After incubation at 37 °C for 10–180 min, the reactions were stopped by the addition of 1 ml of ice-cold trichloroacetic acid (10%, w/v). After 16 h at 4 °C the precipitate was recovered by filtration on Whatman GF/C glass microfibre discs and washed with 3 ml of ice-cold trichloroacetic acid (10%, w/v) followed by 3 ml of ice-cold acetic acid (1%, v/v). The discs were air-dried, placed in scintillant and counted for radioactivity in a liquid scintillation counter.

Assay B. To measure the transfer of [¹⁴C]galactose from UDP[¹⁴C]galactose to endogenous acceptor proteins, the method of Grab *et al.* (1984) was modified as follows. Cells [(1–3) $\times 10^8$] were lysed in 100 μ l of sodium acetate buffer (50 mM, pH 6.5) containing 0.15 mM-UDPGalactose, 0.05 μ Ci of UDP[¹⁴C]galactose, 5 mM-MnCl₂, 5 mM-MgCl₂, 10 mM-ATP, 1 mM-dithiothreitol, 0.02% (w/v) Na₂S₂O₃ and protease inhibitors (chymostatin, leupeptin and pepstatin; each at 1 μ M). In the appropriate experiments 0.1% or 1.6% (v/v) Triton X-100 and ovalbumin (1%, w/v) were added. After a 10–60 min incubation at 37 °C the reaction was stopped and labelled proteins were counted for radioactivity as described in assay A.

Incorporation assay. For SDS/PAGE, samples were prepared according to assay A or assay B using radioactive UDP[¹⁴C]galactose (0.125 μ Ci). Since phosphodiesterases appear to be very active in trypanosome lysates, the included ATP concentration (10 mM) minimized unspecific UDP[¹⁴C]galactose cleavage. Following a 120 min incubation period at 37 °C, 50 μ l aliquots of the lysates were diluted with the same volume of 2-fold concentrated SDS sample buffer (Laemmli, 1970). Additionally, 40 μ l aliquots of the lysates were precipitated with 1 ml of ice-cold trichloroacetic acid (10%, w/v) and prepared for liquid scintillation counting as described in assay A. Because of the lowered substrate concentration (diluted up to 1:40), the enzyme reaction was not linear for 120 min. Thus, although a high incorporation of radiolabelled galactose occurs, the actual

values, as monitored by liquid scintillation counting, do not reflect specific enzyme activities (compare data in Table 2 and Fig. 1b). We therefore use the dimension pmol/mg of protein, rather than pmol/min per mg of protein as in the enzyme assay.

Biosynthetic labelling of trypanosomes

Isolated trypanosomes (10⁸ cells) were labelled *in vivo* with [³⁵S]methionine as described by Duszenko *et al.* (1988). Cells were preincubated (3 $\times 10^7$ cells/ml) for 30 min at 37 °C in the presence or absence of 0.8 μ g of tunicamycin/ml before the addition of [³⁵S]methionine (10 μ Ci/ml). After a 60 min incubation period, cells were washed four times in minimum essential medium in the presence or absence of tunicamycin, and then immunoprecipitations were performed.

Preparation of antibodies and immunoprecipitation

Polyclonal antibodies were isolated from rabbit antisera raised against purified MITat 1.4 sVSG or MITat 1.5 sVSG by affinity chromatography using purified antigens linked to CNBr-activated Sepharose 4B (Pharmacia, Freiburg, Germany) (Ferguson *et al.*, 1986). The pooled antibody fractions were dialysed against distilled water and lyophilized. The dried antibodies (2 mg/ml of gel) were coupled to CNBr-activated Sepharose 4B according to the manufacturers' instructions and stored at 4 °C in 10 mM-sodium phosphate buffer (pH 7.4) containing 120 mM-NaCl, 10 mM-KCl and 0.05% (w/v) Na₂S₂O₃. For [¹⁴C]galactose labelling experiments, (2–5) $\times 10^8$ cells were preincubated for 5 min at 37 °C in 50 mM-sodium acetate buffer (see assay B) without radioactive and non-radioactive sugar donors. After centrifugation (13000 g, 1 min), supernatants containing VSG molecules released from the surface of trypanosomes were discarded. Pellets were resuspended in sodium acetate buffer (2 $\times 10^8$ cells/100 μ l) and incubated (37 °C, 120 min) in the presence of radioactive UDP[¹⁴C]galactose (0.125 μ Ci/100 μ l). For immunoprecipitation, samples were centrifuged (13000 g, 1 min) and then resuspended in 1 ml of 50 mM-Hepes buffer A (pH 7.2) containing 0.5% (v/v) Nonidet P-40, 0.1% (w/v) sodium deoxycholate, protease inhibitors (chymostatin, leupeptin and pepstatin; each at 1 μ M) and 0.1% (w/v) BSA. A 1:1 slurry of Sepharose 4B-coupled antibodies (100 μ l) was added and the mixture was rotated end-over-end for 30 min. The precipitate was washed once in 1 ml of Hepes buffer B [containing 0.1% (w/v) BSA and 0.5 M-NaCl] and twice in 1 ml of Hepes buffer C (without BSA and NaCl), and finally dissolved by boiling for 5 min in 60–110 μ l of 1% (w/v) SDS. Aliquots of [³⁵S]methionine-labelled cells (5 $\times 10^6$ cells) were precipitated in 1 ml of Hepes buffer A with 40 μ l of a 1:1 slurry of Sepharose 4B-coupled antibodies and prepared as described above.

Release of *N*-linked oligosaccharides

Release of *N*-linked oligosaccharides was performed in a total volume of 200 μ l. Aliquots of 20 μ l of immunoprecipitated VSG (in 1% SDS) or 20 μ l of [¹⁴C]galactose-labelled trypanosomal lysate (adjusted to 2% SDS and boiled for 2 min) were added to 0.25 M-sodium phosphate buffer (pH 7.5) containing 10 mM-EDTA, 1.2% (w/v) *n*-octyl glucoside, 1 mM-2-mercaptoethanol and protease inhibitors (chymostatin, leupeptin and pepstatin; each at 1 μ M). The reactions were started by addition of 2 units of PNGase F, and samples were incubated for 18 h at 37 °C. Protein was precipitated in the presence of deoxycholate and trichloroacetic acid according to the method of Bensadoun & Weinstein (1976) prior to SDS/PAGE separation and fluorography. PNGase F-treated samples, diluted to 1 ml with distilled water, were incubated for 15 min at room temperature with 8 μ l of sodium deoxycholate (2%, w/v) and insulin (50 μ g/ml) as carrier. After addition of 100 μ l of trichloroacetic acid (67%,

w/v), centrifugation (30 min, 15000 *g*, 4 °C) yielded protein pellets which were resuspended in 20 μ l of SDS sample buffer and neutralized using NH_4OH (8% v/v).

Exoglycosidase digestions

[^{14}C]Galactose-labelled VSG was treated with coffee-bean α -galactosidase (6 units) or bovine testes β -galactosidase (26 munits) in a total volume of 200 μ l for 18 h at 37 °C. To 20 μ l of immunoprecipitated VSG (in 1% SDS), either (1) 0.1 M-sodium phosphate buffer, pH 6.5 (α -galactosidase) or (2) 0.1 M- Na_2HPO_4 , titrated to pH 4.3 with 0.1 M-citric acid and containing 1.2% (w/v) *n*-octyl glucoside to neutralize the denaturing effect of SDS (β -galactosidase), was added. Following the incubation period, exoglycosidases were found to be at least 80% active, as determined with *p*-nitrophenyl α -D-galactopyranoside or *p*-nitrophenyl β -D-galactopyranoside (20 mM) as substrate. Digests were prepared for SDS/PAGE as described for PNGase F treatment.

PAGE and fluorography

Lysates of [^{14}C]galactose-labelled trypanosomes [(1–5) $\times$ 10⁷ cell equivalents] or immunoprecipitates thereof were analysed by discontinuous SDS/PAGE (Laemmli, 1970) using 10% running gels. The gels were soaked in Amplify (Amersham-Buchler), dried and exposed to Kodak XAR-5 film at –70 °C.

Protein estimation

Protein content was estimated by its ability to bind Coomassie Blue, using a commercial kit (Bio-Rad Laboratories, München, Germany). Samples of 10⁷ trypanosomes were completely solubilized in 200 μ l of 0.05 M- NaOH and stored for 16 h at 4 °C prior to measurements. BSA was used as reference protein.

RESULTS

Enzymic measurements of β -galactosyltransferase

Galactosyltransferase activity was initially measured in cell lysates using an enzyme assay (assay A) adopted from Steiger *et al.* (1980) to measure *N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase activity. UDP[^{14}C]galactose is transferred either to endogenous acceptor proteins, abundantly present in the cell lysate, or to appropriate exogenous proteins, such as ovalbumin or isolated sVSG. Using trypanosome clones MITat 1.4 and MITat 1.5, which contain inversely galactosylated VSG molecules (Table 1), we found galactosyltransferase activities of 2.7 pmol/min per mg of protein in clone MITat 1.5 and 0.2 pmol/min per mg in clone MITat 1.4 (Table 2). Since we could not rule out the possibility that a suitable acceptor protein

was missing in MITat 1.4 lysates, the experiments were repeated using ovalbumin, a glycoprotein which contains terminal *N*-acetylglucosamine residues and is thus often used as a substrate for *N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase (Schanbacher & Ebner, 1970). However, even when using ovalbumin as an external galactose acceptor, galactosyltransferase activity in this clone remained at a low level (Table 2). Since the VSG of clone MITat 1.4 contains galactose exclusively in α -glycosidic linkages in the GPI anchor, we were prompted to test whether galactosyltransferase activity could be increased by using a putative galactose acceptor for these α -galactosyltransferases. We therefore used sVSG isolated from clone MITat 1.5, which may possibly serve as a substrate, since the core structure of its GPI anchor seems virtually identical to that of MITat 1.4, lacking only the galactose residues. However, as shown in Table 2, addition of sVSG MITat 1.5 had a negligible effect on galactosyltransferase activity.

In order to determine whether expression of galactosyltransferase activity corresponds to the VSG galactosylation pattern, we used three additional clones: MITat 1.6, in which the VSG molecule is galactosylated in the GPI anchor but not in the *N*-glycan; MITat 1.2, whose VSG is galactosylated at both sites; and AnTat 1.8, whose VSG is immunologically cross-reactive with MITat 1.4 VSG and probably expresses the same antigen type, but is derived from a different strain (Cross, 1977; Matthysens *et al.*, 1981). Interestingly, both clones showing the same galactosylation pattern of their respective VSGs as MITat 1.4 expressed an invariably low galactosyltransferase activity (0.2–0.6 pmol/min per mg) whatever acceptor protein was used (Table 2). In contrast, clone MITat 1.2, which contains galactose in the anchor as well as in internal *N*-glycans, showed a galactosyltransferase activity of 1.3 pmol/min per mg of protein (Table 2).

Additionally, we tested whether galactosyltransferase activity is differentially expressed during the life cycle of the parasite. For this purpose, bloodstream forms of clone MITat 1.4 were transformed to procyclic mid-gut forms, which lack the surface coat and do not synthesize VSG, by applying a conventional transformation protocol as previously described (Hamm *et al.*, 1990). Since VSG is missing in procyclic forms, ovalbumin was used as an acceptor protein in these experiments. However, as shown in Table 2, galactosyltransferase activity in these forms remained at a low level.

Labelling of endogenous protein acceptors *in vitro* via UDP[^{14}C]galactose

In order to measure the transfer of [^{14}C]galactose to protein acceptors using enzyme assay A, trypanosome lysates were electrophoretically separated by SDS/PAGE and analysed by fluorography. Additionally, aliquots of the lysates were subjected to trichloroacetic acid precipitation in order to quantify the amount of labelled protein by liquid scintillation counting. Fluorography of the corresponding acrylamide gels revealed that, in clone MITat 1.4, no incorporation of radiolabelled galactose was detectable into endogenous trypanosomal proteins, including VSG (Fig. 1*a*, lane 1), whereas a minor labelling of ovalbumin occurred upon addition of this exogenous acceptor protein (Fig. 1*a*, lane 3). In accordance with these results, galactose labelling of total trichloroacetic acid-precipitable material was rather low: 12 pmol/mg without exogenous proteins and 20 pmol/mg in the presence of ovalbumin (Fig. 1*b*). In contrast, with clone MITat 1.5, a spectrum of different trypanosome proteins (molecular masses of 35, 33, 21 and 19 kDa), including VSG (60 kDa), was labelled (Fig. 1*a*, lane 2). Addition of ovalbumin to these lysates led to a slight increase in [^{14}C]galactose incorporation (51 pmol/mg versus 37 pmol/mg; Fig. 1*b*). However, as judged by fluorography, the exogenous acceptor

Table 1. Galactosylation of VSG molecules from *T. brucei*

Compositional data are from: Holder (1985)^a, Holder & Cross (1981)^b and Ferguson *et al.* (1988)^c. AnTat 1.8 serologically cross-reacts with antigen MITat 1.4 (Cross, 1977).

<i>T. brucei</i> clone	Synonym	Galactosylation	
		<i>N</i> -Glycan	GPI anchor
MITat 1.5	118	+ ^a	– ^a
MITat 1.4	117	– ^b	+ ^c
MITat 1.6	121	– ^b	+ ^b
MITat 1.2	221	+ ^b	+ ^b
AnTat 1.8	117	–	+

Table 2. Galactosyltransferase activities in *T. brucei* (assay A)

Trypanosome lysates were incubated according to enzyme assay A in the presence of 1.6% (v/v) Triton X-100. The final concentrations of added acceptor proteins were: ovalbumin, 1% (w/v); MITat 1.5 sVSG, 0.1% (w/v). The values are means $\pm$ s.d. for (*n*) individual experiments. MITat 1.4P indicates procyclic forms of clone MITat 1.4; n.d., not determined.

<i>T. brucei</i> clone	Endogenous protein	Specific activity (pmol/min per mg of protein)	
		Exogenous acceptor	
		Ovalbumin	MITat 1.5 sVSG
MITat 1.5	2.7 $\pm$ 1.3 (11)	3.3 $\pm$ 1.8 (9)	—
MITat 1.4	0.2 $\pm$ 0.1 (2)	0.4 $\pm$ 0.2 (10)	0.2 $\pm$ 0.1 (2)
MITat 1.6	0.3 $\pm$ 0.2 (2)	0.5 $\pm$ 0.1 (2)	0.5 $\pm$ 0.0 (2)
MITat 1.2	1.3 $\pm$ 0.5 (2)	n.d.	n.d.
AnTat 1.8	0.2 $\pm$ 0.2 (2)	0.6 $\pm$ 0.1 (2)	0.3 $\pm$ 0.4 (2)
MITat 1.4P	n.d.	0.6 $\pm$ 0.2 (2)	n.d.

Fig. 1. Transfer of [14 C]galactose to trypanosomal proteins and ovalbumin

(a) Trypanosome lysates of clone MITat 1.4 or MITat 1.5 were incubated according to enzyme assay A. Incubation was carried out in the presence (+) or absence (—) of ovalbumin (1%, w/v) and/or BmGT (1 munit). [14 C]Galactose acceptors were separated by SDS/PAGE and identified by fluorography. In lane 9, BmGT and ovalbumin were incubated without trypanosomal lysate. (b) Trypanosome lysates of clone MITat 1.4 ($\square$) or MITat 1.5 ($\boxplus$) were incubated according to enzyme assay A. Incubation was performed as follows: 1, no ovalbumin (Ov) or BmGT; 2, +ovalbumin (no BmGT); 3, +ovalbumin + BmGT; 4, +BmGT (no ovalbumin). Aliquots of the lysates were precipitated with trichloroacetic acid and analysed by liquid scintillation counting to calculate [14 C]-galactose incorporation.

protein was only faintly labelled and was thus unable to compete efficiently with trypanosomal proteins for galactosylation (Fig. 1a, lane 4). These results (i.e. labelling of MITat 1.5 VSG but not

Fig. 2. Galactosylation of isolated proteins by BmGT

BmGT (10 munits) was incubated with the following acceptor proteins (each 0.1%, w/v) according to enzyme assay A: lane 1, ovalbumin (Ov); lane 2, MITat 1.4 sVSG; lane 3, MITat 1.5 sVSG.

MITat 1.4 VSG) are readily explained by the assumption that the enzyme assay used in these experiments detects galactosyltransferase activities acting on *N*-glycans but not at the GPI anchor. Labelling of ovalbumin suggests the presence of glycopeptide β 1,4-galactosyltransferase. Galactosyltransferases detectable in assay A seem to be more active in clones in which the VSG is galactosylated in internal glycans (MITat 1.2 and MITat 1.5) than in clones where the VSG contains galactose exclusively in the GPI anchor (MITat 1.4, MITat 1.6 and AnTat 1.8).

The suitability of the enzyme assay for detection of glycopeptide β 1,4-galactosyltransferase activity was demonstrated by incubation of commercially available BmGT and ovalbumin (Fig. 1a, lane 9). Addition of both BmGT and ovalbumin to lysates of either clone MITat 1.4 or clone MITat 1.5 led to a considerable increase in radiolabelling (215 pmol/mg and 242 pmol/mg respectively), whereby ovalbumin was the primary protein acceptor (Fig. 1a, lanes 5 and 6; Fig. 1b). However, if BmGT was added to MITat 1.4 lysates in the absence of ovalbumin, the enzyme only faintly labelled a 74 kDa endogenous protein (Fig. 1a, lane 7), and [14 C]galactose incorporation into total trichloroacetic acid-precipitable material did not increase significantly (16 pmol/mg) as compared with the control experiment (12 pmol/mg) (Fig. 1b). An inhibitory effect of the lysate on BmGT activity was ruled out, since BmGT activity was 580 pmol/min per ml in the absence and 600 pmol/min per ml in the presence of trypanosome lysate, using ovalbumin as a substrate. Addition of BmGT to lysates of clone MITat 1.5 led to a nearly 2-fold increase in [14 C]galactose incorporation into trichloroacetic acid-precipitable proteins (69 pmol/mg versus 37 pmol/mg respectively; Fig. 1b), but the labelling pattern of galactosylated endogenous proteins remained the same as in trypanosome lysates alone, apart from an

Fig. 3. PNGase F treatment of [^{14}C]galactose-labelled lysates in *T. brucei* clone MITat 1.5

Trypanosome lysates were incubated according to enzyme assay A (lanes 1 and 2) or enzyme assay B (lanes 3 and 4). Lysates were PNGase F-treated (lanes 2 and 4) or untreated (lanes 1 and 3), and release of *N*-linked glycans from glycoproteins was shown by fluorography of SDS/polyacrylamide gels. The arrows indicate glycosylated and non-glycosylated VSG.

Fig. 4. Effect of Triton X-100 on [^{14}C]galactose labelling in lysates of *T. brucei* clone MITat 1.4

Trypanosome lysates were incubated according to enzyme assay B in the presence of ovalbumin (Ov; 1%, w/v), and [^{14}C]galactose transfer to acceptor proteins was visualized by fluorography. Triton X-100 concentrations were: lane 1, none; lane 2, 0.1% (v/v); lane 3, 1.6% (v/v).

additional galactosylated 74 kDa protein (Fig. 1a, compare lanes 8 and 2). We further showed that isolated sVSG from MITat 1.5, but not sVSG from MITat 1.4, could serve as a protein acceptor molecule when incubated with BmGT and UDP[^{14}C]galactose in this enzyme assay (Fig. 2).

Further evidence for *N*-galactosylation of endogenous proteins in assay A *in vitro* was obtained by PNGase F digestion of [^{14}C]galactose-labelled proteins of clone MITat 1.5. This enzyme, with broad specificity for internal glycans of glycoproteins (Tarentino *et al.*, 1985), released the [^{14}C]galactose label of all endogenous proteins, including MITat 1.5 VSG (Fig. 3, lanes 1 and 2).

Evidence for galactosyltransferase activity acting on the VSG membrane anchor

Since MITat 1.4 VSG was not labelled at all in the experiments described above, we concluded that GPI-anchor-modifying α -galactosyltransferase activities, necessarily present in clone

Fig. 5. Incorporation of [^{14}C]galactose into trypanosomal proteins: effect of incubation conditions

Trypanosome lysates of clones MITat 1.4, MITat 1.5, MITat 1.2, AnTat 1.8 and MITat 1.6 were incubated according to either enzyme assay A (lanes 2, 4, 6, 8 and 10) or enzyme assay B (lanes 1, 3, 5, 7 and 9). [^{14}C]Galactose acceptor proteins were identified by fluorography of SDS/polyacrylamide gels.

MITat 1.4, must be destroyed or inhibited in the enzyme assay used. We therefore modified the lysis and assay conditions in order to detect α -galactosyltransferase activity, using MITat 1.4 VSG labelling as an indicator, because it contains galactose in α -glycosidic linkages only and obviously cannot serve as protein acceptor for internal glycan-modifying enzymes. Instead of lysing the cells by addition of detergent (1.6% Triton X-100), as in the case of assay A, an osmotic lysis was used in assay B. Galactosyltransferase activity was measured as before by transfer of [^{14}C]galactose via UDP[^{14}C]galactose to endogenous acceptor proteins. The galactosyltransferase activity of clone MITat 1.4 was 3.5 pmol/min per mg of protein, i.e. about 15-fold greater than in assay A. Fluorography of the corresponding acrylamide gels revealed that VSG MITat 1.4 was, along with other endogenous trypanosomal proteins (molecular masses 92, 74, 66, 50, 35, 33, 26 and 24 kDa), effectively galactosylated (Fig. 4, lane 1). As shown in Fig. 4 the concentration of Triton X-100 is of critical importance since, as in assay A, galactosyltransferase activity was effectively inhibited in the presence of 1.6% Triton X-100, whereas ovalbumin was still radiolabelled (Fig. 4, lane 3). Interestingly, at low detergent concentrations (0.1% Triton X-100), VSG and other trypanosomal proteins showed a decreased incorporation rate (Fig. 4, lane 2). Preliminary results show that other clones, in which membrane-anchor-specific galactosyltransferase activities are expected (MITat 1.2, MITat 1.6 and AnTat 1.8) also had considerably increased galactosyltransferase activities in assay B (ranging from 8 pmol/min per mg to 14 pmol/min per mg), whereas the specific activity in clone MITat 1.5 increased only slightly (3.7 pmol/min per mg, compared with 2.7 pmol/min per mg in assay A).

A comparison of the different galactosylation patterns in the two assays is shown in Fig. 5. In all clones investigated so far, an osmotic lysis led to the galactosylation of the respective VSG molecule as well as of some other endogenous proteins, especially those of 92, 88, 74, 66, 50, 47, 35 and 33 kDa, which appeared regularly as galactose acceptors. On the other hand, in assay A a significant labelling of VSG was only detectable in clones MITat 1.5 and MITat 1.2, which both contain complex-type oligosaccharides in their respective VSG molecules. Based on these results, we propose that VSG labelling of clones MITat 1.4, MITat 1.6 and AnTat 1.8 reveals galactosyltransferase activities which modify the VSG membrane anchor; these are detectable in assay B but not in assay A.

Incorporation of [14 C]galactose into the GPI anchor of MITat 1.4 VSG

Osmotic lysates of clone MITat 1.4 were subject to immunoprecipitation using polyclonal rabbit antibodies raised against purified MITat 1.4 sVSG. As shown by fluorography (Fig. 6, lane 3), immunoprecipitated MITat 1.4 VSG ran on SDS/PAGE as a single band with a molecular mass of 60 kDa. PNGase F treatment of immunoprecipitated MITat 1.4 VSG led to a VSG molecule with a molecular mass of 58 kDa on SDS/PAGE (Fig. 6, lane 4), which is consistent with the degradation of the one internal *N*-glycan. Identical VSG molecules with molecular masses of 60 and 58 kDa respectively (Fig. 6, lanes 1 and 2) were obtained upon metabolic labelling using [35 S]methionine in the presence and the absence of tunicamycin, an antibiotic that inhibits *N*-glycosylation, but not the addition of the GPI anchor (Ferguson *et al.*, 1986). [14 C]Galactose labelling of MITat 1.4 VSG was resistant to PNGase F treatment, as validated by gel fluorography (Fig. 6, lanes 3 and 4), thus providing further evidence for galactosylation of the GPI anchor *in vitro* in assay B. This galactosylation was due to α -galactosyltransferase activity, as shown by exoglycosidase digestion; treatment of immunoprecipitated MITat 1.4 VSG with α -galactosidase led to a complete release of [14 C]galactose labelling (Fig. 6, lane 5), whereas the labelling was resistant to β -galactosidase digestion (Fig. 6, lane 6).

Immunoprecipitated and PNGase F-digested MITat 1.5 VSG showed a shift in molecular mass from 60 kDa to 55 kDa (Fig. 6, lanes 7 and 8), due to digestion of the three *N*-glycosylation sites in this VSG molecule (Holder, 1985; Ferguson *et al.*, 1986). Although [14 C]galactose labelling of VSG was decreased, it did not disappear completely with the removal of *N*-glycans (Fig. 6, lane 8). This result might be indicative of a minor galactosylation of the MITat 1.5 VSG GPI anchor, as proposed recently (Mayor *et al.*, 1990). Surprisingly, exoglycosidase digestions of immunoprecipitated MITat 1.5 VSG revealed that only some of the [14 C]galactose labelling was susceptible to β -galactosidase (Fig. 6, lane 10), whereas a major proportion of galactosylation *in vitro* obviously occurred by the action of α -galactosyltransferases, since VSG labelling was undetectable after α -galactosidase digestion (Fig. 6, lane 9). This result was unexpected, as galactose residues were thought to be β -glycosidically linked in internal glycans. However, a recent study indeed revealed that clone MITat 1.5 and others contain a significant proportion of terminal α 1,3-linked galactose residues (Zamze *et al.*, 1991).

PNGase F treatment of osmotic lysates from MITat 1.4 and

Fig. 6. Incorporation of [14 C]galactose into the GPI anchor of MITat 1.4 VSG and MITat 1.5 VSG

Trypanosome lysates of clones MITat 1.4 and MITat 1.5 were incubated with UDP[14 C]galactose according to enzyme assay B. Immunoprecipitated MITat 1.4 VSG (1.8×10^8 cell equivalents) was either untreated (lane 3) or treated with PNGase F (lane 4), α -galactosidase (lane 5) or β -galactosidase (lane 6). Immunoprecipitated MITat 1.5 VSG (1.4×10^8 cell equivalents) was untreated (lane 7) or treated with PNGase F (lane 8), α -galactosidase (lane 9) or β -galactosidase (lane 10), parallel to clone MITat 1.4. As a control, cells of clone MITat 1.4 were metabolically labelled using [35 S]methionine and immunoprecipitated in the presence (lane 2) or absence (lane 1) of tunicamycin. Arrows (MITat 1.5) (MITat 1.4) and arrowheads indicate glycosylated and non-glycosylated VSG.

MITat 1.5 also led to the total release of [14 C]galactose labelling in proteins other than VSG (Fig. 3, lanes 3 and 4). Thus all other trypanosomal proteins except VSG were galactosylated only within their *N*-glycans in assay B.

DISCUSSION

In most VSG molecules analysed to date the GPI membrane anchor is substituted with α -linked galactose residues, but the corresponding α -galactosyltransferases have not been demonstrated so far. Using UDP[14 C]galactose as an activated sugar donor, we have tried to identify the galactosyltransferase activity modifying the GPI anchor by analysing the radiolabelled products fluorographically. Since MITat 1.4 VSG does not contain galactose in its *N*-glycans and could not be galactosylated by the commercially available BmGT *in vitro*, galactosylation of this antigen type was taken to indicate anchor-modifying galactosyltransferase activity. Two enzyme assays (referred to as assays A and B) have been applied which differ mainly in their detergent content.

Using assay A, significant VSG labelling was obtained only in those clones which contain complex-type oligosaccharides (MITat 1.5 and MITat 1.2). In contrast, no labelling was observed in clones MITat 1.4, MITat 1.6 or AnTat 1.8, which contain galactose in their GPI membrane anchor but not in their internal glycans. Addition of ovalbumin led to a slight increase in galactosyltransferase activity in all clones, and a faint labelling of this acceptor protein was seen upon fluorographic analysis of clones MITat 1.4 and MITat 1.5. Hence ovalbumin seems to be a suitable substrate for trypanosomal *N*-acetylglucosaminyl glycopeptide β 1,4-galactosyltransferase, but cannot efficiently compete with endogenous substrates. Assay A was originally designed to detect glycopeptide β 1,4-galactosyltransferase activity in trypanosomes, and PNGase F treatment of clone MITat 1.5 lysates indeed revealed that only *N*-linked glycans could be labelled after detergent lysis of cells. Recently, however, structural data of *N*-linked oligosaccharides have become available which demonstrate terminal α 1,3-linked galactose residues (Zamze *et al.*, 1991). We cannot rule out the possibility that the respective α -galactosyltransferase may be active in assay A as well, and this may indeed account for the faint labelling of exogenous ovalbumin. However, our results clearly show inhibition of α -galactosyltransferases acting on the VSG membrane anchor.

Interestingly, Triton X-100-insensitive enzyme activities are very low in those clones which do not contain galactosylated *N*-glycans in their VSG molecules (MITat 1.4, MITat 1.6 and AnTat 1.8); medium in clone MITat 1.2; and very high in a clone with VSG molecules exclusively galactosylated in its internal glycans (MITat 1.5). Thus it is tempting to speculate that the terminal glycosylation sequences of VSG *N*-glycans reflect the activity of the corresponding galactosyltransferases. As shown previously in mammalian cells, terminal *N*-glycosylation sequences are differentially expressed and are subject to change during development, differentiation and oncogenic transformation (reviewed in Rademacher *et al.*, 1988). In our study, galactosyltransferase activity was unaffected upon differentiation of clone MITat 1.4 bloodstream forms to procyclic forms *in vitro*, but was obviously changed in different clones derived from the same strain. Thus galactosyltransferase activity seems to be affected by antigenic variation. Since expression-site associated genes are transcribed co-ordinately with the respective VSG gene (Cully *et al.*, 1985), some of these gene products might be related to VSG processing.

On changing the experimental conditions to an enzyme assay initially adopted from published data (Grab *et al.*, 1984), galactosyltransferase activity increased considerably in clones

MITat 1.2, MITat 1.4, MITat 1.6 and AnTat 1.8, and slightly in clone MITat 1.5. Fluorography of trypanosomal proteins labelled via UDP[¹⁴C]galactose showed a dominant VSG labelling in all clones, which was confirmed by immunoprecipitation of MITat 1.4 VSG and MITat 1.5 VSG. Since MITat 1.4 VSG was radiolabelled in assay B, it seems likely that galactosyltransferases modifying the membrane anchor are active under these conditions. We were thus prompted to determine if radioactive galactose is indeed linked to the GPI anchor of MITat 1.4 VSG, as would be expected. Treatment of immunoprecipitated MITat 1.4 VSG with PNGase F led to a VSG molecule of a smaller molecular mass, consistent with the loss of the *N*-glycan, whereas the labelling remained intact. Additionally, exoglycosidase digestion revealed α -linked galactose residues, and finally structural analysis of the isolated VSG membrane anchor has been performed in collaboration with Dr. M. A. J. Ferguson (University of Dundee, Dundee, Scotland). The preliminary data clearly show predominant or exclusive galactosylation of the membrane anchor, whereby the major peak co-chromatographed exactly with authentic Man α 1,2-Man α 1,6(Gal α 1,3)Man α 1,4-2,5-anhydromannitol (results not shown). Interestingly, PNGase F-treated MITat 1.5 VSG, a variant containing a non-galactosylated membrane anchor (Holder, 1985), showed decreased but still detectable radioactive labelling. This result is consistent with recent findings from Mayor *et al.* (1990), who observed microheterogeneity, with a minimum of galactosylation, when they analysed the neutral glycan derived from the GPI anchor of MITat 1.5 VSG. A partial decrease in MITat 1.5 VSG labelling due to PNGase F digestion suggests galactosylation of *N*-glycans in this clone. Using assay B, a spectrum of at least eight trypanosomal proteins beside VSG appeared regularly as galactose acceptors. PNGase F digestions of MITat 1.4 and MITat 1.5 lysates revealed galactose labelling exclusively to *N*-glycans in all proteins apart from VSG. These galactosyltransferases are sensitive to Triton X-100, since even low detergent concentrations led to decreased [¹⁴C]galactose incorporation rates. In contrast, Steiger *et al.* (1980) found that 1.6% Triton X-100 was essential for complete activation of β 1,4-galactosyltransferase, and BmGT did not lose activity at this detergent concentration (results not shown).

On comparing both assay procedures, we were able to show that detergent lysis inhibits galactosylation of the membrane anchor in assay A. Omitting Triton X-100 from assay A led to similar activities and labelling patterns as were obtained using optimized assay B (results not shown), and addition of 1.6% Triton X-100 to assay B resulted in a nearly complete inhibition of galactosyltransferase activity in clone MITat 1.4. It seems likely that galactosylation of the membrane anchor depends on membrane integrity for the correct presentation of the substrate or for correct conformation of the enzymes, and is thus readily observed upon hypo-osmotic lysis but not after detergent lysis. The galactose side-branch of the GPI anchor was suggested to have a possible space-filling function for maintenance of the coat integrity (Homans *et al.*, 1989). Insertion of VSG into membranes may therefore be important for recognition by the modifying α -galactosyltransferases. There is now clear evidence that the GPI anchor is built up from common precursor glycolipids in trypanosomes which are probably transferred to the VSG molecule (Krakow *et al.*, 1986; Menon *et al.*, 1988; Doering *et*

al., 1990). Since most of the anchor precursors seem not to be galactosylated (Mayor *et al.*, 1990), it would be interesting to learn where α -galactosyltransferases are located to within the cell, in order to study these reactions in more detail.

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