

International Journal for Parasitology 33 (2003) 47-56



Mucin-type *O*-glycosylation in *Fasciola hepatica*: characterisation of carcinoma-associated Tn and sialyl-Tn antigens and evaluation of UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase activity

Teresa Freire^a, Cecilia Casaravilla^b, Carlos Carmona^b, Eduardo Osinaga^{a,*}

^aDepartamento de Bioquímica, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay ^bUnidad de Biología Parasitaria, Departamento de Biología Celular y Molecular, Facultad de Ciencias, Instituto de Higiene, Montevideo, Uruguay

Received 22 July 2002; received in revised form 30 September 2002; accepted 11 October 2002

Abstract

Simple mucin-type cancer-associated O-glycan structures, such as the Tn antigen (GalNAc-O-Ser/Thr), are expressed by certain helminth parasites. These antigens are involved in several types of receptor-ligand interactions, and they are potential targets for immunotherapy. The aim of this work was to study the initiation pathway of mucin-type O-glycosylation in Fasciola hepatica, performing a biochemical and immunohistochemical characterisation of Tn and sialyl-Tn antigens, and evaluating the ppGaNTase activity, which catalyses the first step in O-glycan biosynthesis. Using ELISA, both Tn and sialyl-Tn antigens were detected predominantly in the somatic and deoxycholate extracts. Immunofluorescence analysis revealed that Tn antigen is preferentially expressed in testis, while sialyl-Tn glycoproteins were more widely distributed, being present in parenchymal cells, basal membrane of the tegument, and apical surface of epithelial cells lining the caeca. On the basis of their electrophoretic mobility, Tn glycoproteins were resolved as six components of 10, 37, 76, 125, 170 and 205 kDa, and sialyl-Tn components showed an apparent molecular mass of 28 and 32 kDa, and two broad bands of 90-110 and 170-190 kDa. The observation that only the 76 kDa Tn-glycoprotein remained in the 0.6 N perchloric acid-soluble fraction suggests that it could be a good candidate for mucin characterisation in this parasite. The ppGaNTase activity showed its maximal activity at pH 7–7.5 and 37 °C, showing that Mn²⁺ was the best divalent cation activator. Using a panel of nine synthetic peptides as acceptor substrates, we found that F. hepatica ppGaNTase was able to glycosylate both threonines and serines, the best substrates being the peptides derived from the tandem repeat region of human mucins (MUC2 and MUC6), and from Trypanosoma cruzi and Trypanosoma brucei glycoproteins. The results reported here constitute the first evidence on O-glycosylation pathways in F. hepatica, and may help to identify new biological characteristics of this parasite as well as of the host-parasite relationship. © 2002 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Fasciola hepatica; Helminth parasites; O-Glycosylation; Tn antigen; Sialyl-Tn antigen; UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase

1. Introduction

O-Linked oligosaccharide structures play important functions "in vivo" (Hanisch, 2001), involving several types of receptor–ligand interactions (Brockhausen, 1993), and participating in important biological processes, such as cell differentiation, cancer invasion and viral and bacterial infectivity (Hounsell et al., 1996). These glycans also play a structural role by stabilising the polypeptide to which they are attached as well as by protecting the glycosylated protein from proteolytic degradation (Jentoft, 1990). Certain

* Corresponding author. Departamento de Bioquímica, Facultad de Medicina, Av. Gral. Flores 2125, Montevideo CP 11800, Uruguay. Fax: +598-2-924-95-63.

helminth parasites express O-glycans and mucin-like molecules. For example, the trematode Schistosoma mansoni expresses a Thr/Ser-rich glycoprotein in the epithelial cells of the reproductive tract of female worms (Cummings and Nyame, 1999). Furthermore, the presence of Tn antigen (Thr/Ser-O-GalNAc), one of the most specific human tumour-associated structures (Hakomori, 1989), and expressed in mucin-type O-glycans of cancer cells due to a blockage in mucin chain elongation (Brockhausen, 1999), has been observed both in the schistosomula and in the adult worm (Nyame et al., 1987). Recently, we demonstrated that this antigen and sialyl-Tn (Thr/Ser-O-GalNAc-NeuAc) are expressed by larval and adult tissues of the cestode Echinococcus granulosus, and high levels of circulating Tn antigen were detected in the sera of hydatid patients (Alvarez Errico et al., 2001). Moreover, we screened different worm extracts

E-mail address: eosinaga@fmed.edu.uy (E. Osinaga).

from parasites belonging to both major phyla, the Nematoda and the Platyhelminths, and showed that Tn is present in *Mesocestoides corti, Taenia hydatigena, Nippostrongylus brasiliensis, Toxocara canis* and *Fasciola hepatica* (Casaravilla et al., unpublished data). All these results, showing widespread expression of Tn antigen, strongly suggest that truncated *O*-glycosylation does not constitute an aberrant phenomenon in helminth parasites belonging to the most relevant taxonomic groups.

The UDP-GalNAc:polypeptide N-acetylgalactosaminyltransferase (ppGaNTase) (EC 2.4.1.41) catalyses the first step in the O-glycosylation (Marth, 1996), and it determines the number and position of O-linked sugar chains in a protein. This reaction involves the transfer of a GalNAc residue to the hydroxyl side chain of a serine or threonine residue. To date, nine ppGaNTases have been reported in vertebrates (Clausen and Bennett, 1996; Hanisch, 2001). The free-living nematode Caenorhabditis elegans is the only worm in which ppGaNTases have been characterised. Hagen and Nehrke (1998) isolated 11 ppGaNTases in C. elegans, five of which were able to glycosylate synthetic peptides derived from human mucin motifs. All clones encoded type II membrane proteins that shared 60-80% amino acid sequence similarity with the catalytic domain of mammalian ppGaNTase enzymes.

The digenean trematode *F. hepatica* is the causal agent of fasciolosis, a chronic disease in domestic animals and humans. Although glycoconjugates are commonly regarded as critical molecules in the host–parasite balance, for example through diverting immune responses by the continuous shedding of the glycocalyx (Hanna, 1980), there is a virtual lack of structural information on *F. hepatica* glycans and their synthetic pathways. The aim of this work was to study the initiation pathway of mucin-type *O*-glycosylation in *F. hepatica*, performing a biochemical and immunohistochemical characterisation of Tn and sialyl-Tn antigens and evaluating the ppGaNTase activity in adult flukes.

2. Materials and methods

2.1. Monoclonal antibodies and lectins

The mAb 83D4 (IgM), which specifically binds the Tn antigen (Osinaga et al., 2000), was produced from a mouse immunised with cell suspensions obtained from formalin-fixed paraffin-embedded sections of an invasive human breast cancer (Pancino et al., 1990). Monoclonal antibody was precipitated from ascites fluids by dialysis against demineralised water at 4 °C, dissolved in a small volume of 0.5 M NaCl in PBS, and purified by gel-filtration chromatography on Sephacryl S-200. The mAb B72.3 (IgG₁), which recognises the sialyl-Tn determinant (Kjeldsen et al., 1988), was established by immunising a mouse with human colonic cancer cells (Colcher et al., 1981). This MAb was purified by affinity chromatography using a protein A-

Sepharose column. The biotinylated lectins used, obtained from Sigma, were: isolectin B4 from *Vicia villosa* seeds (VV), which specifically agglutinates Tn-exposed red blood cells; *Helix pomatia* (HPA), reactive with non-substituted GalNAc residues; peanut agglutinin (PNA), specific for the T antigen (Thr/Ser-GalNAc-Gal); and wheat germ agglutinin (WGA), which binds GlcNAc and sialic acid residues.

2.2. Preparation of parasite extracts

2.2.1. Detergent soluble extract

Adult worms were collected from cattle livers at an abattoir and washed several times in 0.01 M phosphate (pH 7.2)/ 0.15 M NaCl (PBS) at 4 °C. The flukes were killed by freezing for 30 min at -20 °C, washed twice with PBS at 4 °C, and drained. One gram wet weight of tissue was incubated in 10 ml of 1% deoxycholic acid in 0.15 M glycine (pH 9.0), 0.5 M NaCl, for 60 min at room temperature, 30 min at 37 °C, and then 30 min at 4 °C. The deoxycholateextracted material was centrifuged at 20,000 × g for 60 min and the supernatant was stored at -80 °C until used.

2.2.2. Somatic extract

Live adult flukes were washed several times with PBS, and then homogenised with PBS in a glass tissue grinder. The homogenate was ultrasonicated on an ice bath for 3 min, in 60 s bursts (20% power) with 30 s pauses, using an ultrasonic homogeniser 4710 (Cole-Palmer Instrument Co.), then centrifuged at $48,000 \times g$ for 1 h and the supernatant stored at -80 °C until used.

2.2.3. Excretion/secretion (E/S) products

Live adult flukes were washed six times in PBS (pH 7.3) and then maintained in RPMI-1640 (pH 7.3) containing 2% glucose, 30 mM HEPES and 25 mg/l gentamycin overnight. The culture medium was removed, centrifuged at 12,000 × g for 30 min and the supernatant was stored at -20 °C until used (Dalton and Heffernan, 1989).

2.3. Quantitation of Tn and sialyl-Tn antigens

Tn glycoproteins were detected in parasite preparations by a double-determinant immunolectin-enzymatic method (CA83.4 assay), devised using the anti-Tn mAb 83D4 as the catcher, and the anti-Tn-reactive isolectin B4 from V. *villosa* as the tracer as described by Osinaga et al. (1996). Sample concentrations of Tn glycoproteins were determined by interpolation against a standard curve performed with asialo-ovine submaxillary mucin, a standard source of Tn. The antigen unit was defined as the amount of CA83.4 reactivity found in 10 ng of desialylated mucin. All experimental samples were processed in duplicate.

Sialyl-Tn antigen was detected in parasite preparations by an ELISA. Microtitre wells were coated with 100 μ l of different concentrations of the parasitic extract diluted in 0.1 M carbonate buffer (pH 9.6) by overnight incubation at room temperature. The wells were washed with 0.1% Tween 20 in PBS and incubated with 200 µl of 1% gelatin in PBS (PBS/G) at 37 °C for 1 h. After three washes wells were incubated with 100 µl of mAb B72.3 (10 µg/ml) at 37 °C for 2 h. Afterwards, wells were incubated with a biotinylated anti-mouse-IgG antibody (Sigma) diluted in 0.5% gelatin, 0.1% Tween 20 in PBS (PBS/TG) for 1 h at 37 °C. Unbound material was then washed off and 100 µl of 1/ 2,000 avidin/peroxidase complex (Sigma) in PBS/TG was added for 1 h at 37 °C. Peroxidase activity was demonstrated by incubation in ABTS [2,2-Azin-bis(3-ethylbenz-thiazoline-6-sulphonic acid)] (3 mg) and 30% hydrogen peroxide (7 µl) in phosphate-citrate buffer, pH 5.0 (10 ml). Sample concentrations of sialyl-Tn glycoproteins were determined by interpolation against a standard curve performed with ovine submaxillary mucin, a standard source of sialyl-Tn. The antigen unit was defined as the amount of reactivity found in 10 ng of mucin. All experimental samples were done in duplicate.

2.4. Perchloric acid fractionation of samples

Fasciola hepatica preparations were subjected to perchloric acid precipitation at 4 °C (0.6 N perchloric acid concentration), as previously described (Alvarez Errico et al., 2001). The perchloric acid-soluble fraction was neutralised with 1.2 M KOH and dialysed against PBS. Tn and sialyl-Tn antigens were quantified as described above.

2.5. Immunohistochemical analysis

Paraffin-embedded sections of *F. hepatica* adult forms were prepared following standard protocols. Sections were deparaffinated and rehydrated and then incubated for 1 h at 37 °C with 10 μ g/ml of mAbs 83D4 or B72.3. Sections were extensively washed with PBS and incubated with an appropriate dilution of secondary anti-mouse Ig-FITC conjugated antibody (1/200, Sigma) for 1 h at 37 °C in the dark. Sections were mounted and contra-dyed in 14% polyvinyl alcohol/2% glycerol and observed in a fluorescence microscope. Negative controls were treated in the same way, with the exception that samples were incubated in PBS instead of mAbs.

2.6. SDS-PAGE and Western blotting

Proteins were resolved by SDS-PAGE (3–15% gradient) under reduction and non-reduction conditions as described previously (Laemmli, 1970) and identified by silver staining according to standard protocols. For Western blot analysis, proteins resolved by SDS-PAGE were transferred onto nitrocellulose sheets (Amersham) according to Towbin et al. (1979) at 60 V for 5 h in 20 mM Tris–HCl (pH 8.3), 192 mM glycine, 10% methanol. Residual protein-binding sites on the paper were blocked by incubation with 3% BSA in PBS overnight at 4 °C. The nitrocellulose was then incubated with 3 μ g/ml of mAbs 83D4 and B72.3 for 3 h at 37 °C. After three washes with PBS containing 0.1% Tween 20 and 1% BSA, the membrane was incubated for 1 h at room temperature with goat anti-mouse immunoglobulin conjugated to peroxidase (1/2,000, Sigma) diluted in PBS 0.3% Tween 20 and 3% BSA, and the reaction was developed with enhanced chemiluminesence (ECL, Amersham).

2.7. Purification of Tn glycoproteins

Parasite components containing Tn were affinity-purified from the deoxycolate preparation using a Sepharose-CNBractivated column with immobilised mAb 83D4. Deoxycolate extracts were incubated by recirculation overnight at 4 °C. The column was washed with PBS containing 0.5 M NaCl, and elution was performed at alkaline pH with 0.1 M triethylamine buffer (pH 10.5). The elution peak was monitored by the CA83.4 assay described above.

2.8. Lectin-binding assays

Preliminary sugar composition of Tn-purified proteins from deoxycolate extract was evaluated by lectin-binding assays according to Alvarez Errico et al. (2001). Microtitre wells were coated with 100 μ l of purified Tn glycoproteins diluted in carbonate buffer at different concentrations. Plates were blocked with 200 μ l of PBS/G at 37 °C for 2 h. After washing with PBS, 0.1% Tween 20, wells were incubated for 2 h at 37 °C with biotinylated lectins (HPA, PNA, VV, WGA) diluted in PBS/TG. Plates were washed and avidin/ peroxidase complex diluted 1/2,000 in PBS/TG was added and incubated for 1 h at 37 °C. Reaction was revealed using ABTS as described above.

2.9. Evaluation of ppGaNTase activity using a multisite substrate acceptor

Ovine submaxillary mucin was purified according to Tettamanti and Pigman (1968), subjected to sequential deglycosylation following the procedure described previously (Mendocino and Sangadala, 1998), and used as a ppGaNTase multisite peptide acceptor substrate. The evaluation of the ppGaNTase activity was done as described before (Yoshida et al., 1997). Briefly, the standard reaction mixture contained 50 mM imidazole-HCl (pH 7.2), 10 mM MnCl₂, 0.5% Triton X-100, 150 µM UDP-(³H)-GalNAc (approximately 80,000 dpm), different concentrations of deglycosylated ovine submaxillary mucin, and deoxycolate extract (approximately 100 µg per reaction), in a final volume of 50 µl. The rate of GalNAc transfer to peptide was measured by incubating the mixture at 37 °C for 2 h. The reaction was terminated by adding 50 µl of 250 mM EDTA. The glycosylated product was separated from unreacted UDP-(³H)-GalNAc on a 1 ml AG1X-8 (Cl⁻ form, Sigma) column with 2.6 ml of water as eluent. The radioactivity was measured by a LS Analyzer Beckman scintillation counter. The enzyme activity was expressed as pmoles of UDP-(³H)-GalNAc transferred per mg of

Amino acid sequences of synthetic peptides used to evaluate the ppGaNTase activity	quences of synthetic peptides used to evaluate the ppGaN	Fase activity

Peptide	Amino acid sequence	Reference
Human MUC1 (a)	PDTRPAPGSTA	Gendler et al., 1990
Human MUC1 (b)	HGVTSAPDTRP	Gendler et al., 1990
Human MUC2 (a)	PTTTPITTTTV	Gum et al., 1994
Human MUC2 (b)	VTPTPTPTGTQT	Gum et al., 1994
Human MUC5B	VLTTTATTPTA	Desseyn et al., 1997
Human MUC6	GTTPPPTTLK	Ho et al., 1995
Trypanosoma cruzi mucin	KPPTTTTTTTKPP	Acosta-Serrano et al., 2001
Trypanosoma brucei mucin	SSLLSSFASSAVG	Nolan et al., 1997
Schistosoma mansoni mucin	ISTSPSPSNITTTT	Menrath et al., 1995

protein and per hour. All experiments were done in triplicate. Reactions lacking deglycosylated ovine submaxillary mucin yielded background values that were averaged for each proteic extract and subtracted from each triplicate value. Error bars indicate standard deviations. The same procedure was carried out for the evaluation of ppGaNTase activity at different pH values and temperatures. In this case $5 \mu g$ of deglycosylated ovine submaxillary mucin was used.

2.10. Evaluation of ppGaNTase activity using different synthetic peptides

O-GalNAc transfer was assayed using synthetic peptides (Bio-synthesis Inc.) derived from the tandem repeat sequences of different human mucins (MUC1 (a), MUC1 (b), MUC2 (a), MUC2 (b), MUC5B and MUC6) and highly-glycosylated proteins derived from parasites (*Trypanosoma cruzi*, *Trypanosoma brucei*, and *S. mansoni*) (Table 1). The ppGaNTase assay was performed as described above, using 2 mM synthetic peptide as acceptor substrate and 5 μ g of deglycosylated ovine submaxillary mucin. All experiments were performed in triplicate. Error bars indicate standard deviations of those experiments done in triplicate.

3. Results

3.1. Detection of Tn and sialyl-Tn antigens in F. hepatica extracts

The presence of Tn and sialyl-Tn determinants in different *F. hepatica* extracts was measured using ELISA, expressing the specific activity of each fraction (antigen units/mg protein). The highest levels of Tn antigen were found in the somatic and deoxycolate extracts (Fig. 1A). Sialyl-Tn was also present, predominantly in the somatic fraction (Fig. 1B). Considering that both antigens are highly expressed on carcinoma-associated glycoproteins soluble in perchloric acid, we evaluated the perchloric acid solubility of *Fasciola* Tn and sialyl-Tn glycoproteins. With this procedure, the majority of proteins present in each extract precipitated, while a fraction of Tn glycoproteins were shown to be soluble in 0.6 N perchloric acid, determining a considerable increase in Tn antigen specific activity. In contrast, almost all glycoproteins bearing sialyl-Tn antigen precipitated with perchloric acid.

3.2. Immunohistochemical analysis

In order to determine the localisation of Tn and sialyl-Tn antigens in *F. hepatica*, an immunofluorescence analysis was performed. The Tn antigen expression was predomi-



Fig. 1. Tn and sialyl-Tn antigen levels in *F. hepatica* extracts. Somatic (Som), deoxycolate (DOC), and E/S fractions were analysed for Tn antigen (CA83.4 assay) (A), and for sialyl-Tn antigen (using mAb B72.3) (B), as described in Section 2. Results were obtained from total fractions (\Box) as well as from the 0.6 N perchloric acid-soluble fraction (\blacksquare). The antigen content is expressed as specific activity (AU/mg protein). All experimental samples were processed in duplicate. Tn and sialyl-Tn antigen specific activities were measured many times, giving very similar results in different *F. hepatica* extract preparations. In this figure results obtained in one representative experiment are shown.



Fig. 2. Immunofluorescence analysis of Tn and sialyl-Tn antigens. Adult forms of *F. hepatica* were incubated with the anti-Tn mAb 83D4 (A), with the antisialyl-Tn mAb B72.3 (C,D), and without primary antibody (negative control), which showed no reactivity (B).

nantly observed in testis but not in the carbohydrate-rich tegument (Fig. 2A). Reactivity against sialyl-Tn glycoproteins was shown to be more widely distributed, being present in parenchymal cells, the basal membrane of the tegument, and the apical surface of epithelial cells lining the caeca (Fig. 2C,D).

3.3. Electrophoretic analysis

SDS-PAGE followed by Western blot analysis in *F. hepatica* deoxycolate extracts was performed to determine the pattern of glycoproteins which express the truncated *O*-glycosylated epitopes. The Tn antigen was identified as a group of six components with relative molecular mobility of 10, 37, 76, 125, 170 and 205 kDa (Fig. 3, lane 1). Tn bearing glycoproteins from deoxycolate extracts were purified by affinity chromatography using immobilised mAb 83D4. Following purification, the six components identified by

Western blot analysis were detected by silver staining in SDS-PAGE (Fig. 3, lanes 2 and 3). A very similar pattern was observed in non-reducing and reducing conditions. The component of 125 kDa migrated slightly more slowly in reducing conditions, suggesting the presence of an intrachain disulphide bond. Interestingly, after perchloric acid precipitation of deoxycolate extracts, only the 76 kDa component remained in the soluble fraction (Fig. 3, lane 4). In contrast, sialyl-Tn glycoproteins appeared as only two components (28 and 170–190 kDa) in non-reducing conditions (Fig. 3, lane 5), and revealed other components of 32 and 90–110 kDa in reducing conditions (Fig. 3, lane 6), indicating that disulphide bonds were established between some components.

3.4. Preliminary analysis of sugar composition

The evaluation of Tn and sialyl-Tn antigens co-expres-



Fig. 3. Electrophoretic analysis. Tn antigen was detected in deoxycolate extracts by immunoblot using the mAb 83D4 (lane 1, reduction condition). Purified Tn glycoproteins were silver stained after SDS-PAGE analysis (lanes 2 and 3, non-reduction and reduction conditions, respectively). Perchloric acid (0.6 N) soluble fraction of purified Tn glycoproteins was silver stained after SDS-PAGE analysis (lane 4). Sialyl-Tn antigen was identified in deoxycolate extracts by immunoblot using the mAb B72.3 (lanes 5 and 6, non-reduction and reduction conditions, respectively). Negative controls were incubated with PBS without the primary antibody, and no reactivity was observed (data not shown).

sion was performed by an ELISA. On wells coated with Tnpurified glycoproteins poor reactivity of anti-sialyl-Tn mAb B72.3 was observed, whereas the material was recognised by mAb 83D4 (Fig. 4A), and B72.3 strongly reacted with ovine submaxillary mucin (data not shown). Lectin-binding assay of Tn-purified glycoproteins revealed the presence of other sugars (Fig. 4B). Tn bearing glycoproteins bound PNA, indicating a co-expression of T-antigen, another incomplete *O*-glycosylated carcinoma-associated structure. WGA and *H. pomatia* reactivity were also observed, indicating the presence of sialic acid and/or GlcNAc as well as GalNAc.

3.5. Characterisation of ppGaNTase activity

This enzyme is the key step in Tn antigen synthesis, as it is responsible for the beginning of the O-glycosylation, catalysing the transfer of a GalNAc to threonine or serine residues on an acceptor polypeptide. Taking into account that a single consensus peptide sequence does not exist for mucin-type GalNAc O-glycosylation of serine/threonine (Van den Steen et al., 1998), we started our study using deglycosylated ovine submaxillary mucin, a ppGaNTase multisite peptide acceptor substrate. A typical saturation curve of ppGaNTase activity was obtained using different deglycosylated ovine submaxillary mucin concentrations, revealing a high activity of this enzyme in F. hepatica extracts (data not shown). The ppGaNTase activity was evaluated at different temperatures (4, 20, 28 and 37 °C) observing the higher activity at 37 °C. The O-GalNAc transferase activity had a broad pH optimum with maximal activity between pH 7.0 and 7.5 (data not shown). The enzyme activity showed ion dependence, Mn²⁺ being the best activator among the different divalent cations tested. The values obtained with the seven cations used are shown in Table 2, subtracting the very low activity observed in their absence.

3.6. 'In vitro' glycosylation of synthetic peptides

Considering that GalNAc-O-linked glycopeptide motifs have not been identified yet in *F. hepatica*, we assessed the ppGaNTase activity on a panel of nine synthetic peptides derived from O-glycosylated proteins from parasites (*T. cruzi*, *T. brucei* and *S. mansoni*), and from human mucins (MUC1, MUC2, MUC5B and MUC6) (Table 1). Fasciola hepatica extracts showed glycosylation preference for the MUC2, MUC6, *T. cruzi* and *T. brucei* peptide sequences, although other peptides were also successful acceptor substrates (Fig. 5). The lower enzymatic activity was observed using peptide sequences corresponding to *S. mansoni* and MUC5B mucins.

4. Discussion

It has been demonstrated that parasite glycoconjugates, particularly those present in the E/S fraction and on the surface, play critical roles in the interaction of helminth parasites with their hosts. For example, they can participate in avoiding the immune response by antigenic mimicry of host components (Damian, 1997), inhibiting T cell proliferation (Persat et al., 1996), inducing effective protection (Ellis et al., 1994), or promoting autoimmune responses (Cummings and Nyame, 1999). Mucin-type *O*-linked oligo-saccharide structures, common constituents of many secretory and cell surface proteins, are characterised by the GalNAc which links the oligosaccharide chain to the polypeptide backbone by means of an *O*-linkage to a serine or threonine residue in the protein (Van den Steen et al., 1998).



Fig. 4. Preliminary analysis of sugar composition of Tn antigen. (A) Expression of sialyl-Tn. ELISA performed on wells coated with optimal dilutions of Tn glycoproteins purified from deoxycolate extract and developed with the anti-Tn mAb 83D4 (◆) and the anti-sialyl-Tn mAb B72.3 (■). Ovine submaxillary mucin was used as positive control for mAb B72.3 reactivity (data not shown). (B) Lectin-binding assays. Carbohydrate moieties present in the Tn bearing glycoproteins purified from deoxycolate extract were investigated by a lectin-binding assay evaluating the reactivity of biotin-labelled lectins (HPA, *Helix pomatia*; PNA, peanut agglutinin; VV, *Vicia villosa*; WGA, wheat germ agglutinin).

Recently, we obtained the first evidence about the presence of incomplete *O*-glycosylation (Tn antigen) in the cestode *E. granulosus* (Alvarez Errico et al., 2001), and in other species belonging to the main two phyla of parasitic

Table 2

Evaluation of divalent cation dependence of F. hepatica ppGaNTase act	tiv
ity	

Cation (10 mM)	Transferase activity (pmol/mg.h)	Relative activity (%)
Mn ²⁺	0.910 ± 0.014	100
Cu ²⁺	0.765 ± 0.035	84
Ni ²⁺	0.66 ± 0.10	73
Mg ²⁺	0.625 ± 0.021	69
Zn^{2+}	0.61 ± 0.22	66
Co ²⁺	0.55 ± 0.10	60
Ca ²⁺	0.39 ± 0.05	43



Fig. 5. Evaluation of ppGaNTase activity using synthetic peptide acceptors. Nine synthetic peptides (2 mM) were used to assess the ppGaNTase activity in the conditions described in Section 2. Results were compared to those obtained with deglycosylated ovine submaxillary mucin (5 μ g).

helminths, the Nematoda and the Platyhelminths, including F. hepatica (Casaravilla et al., unpublished data). There is virtually no information available about the structure and synthesis of O-glycans in F. hepatica. In this work we have shown that Tn antigen is present in both somatic and deoxycolate F. hepatica preparations, and, at lower levels, in the E/S products. Sialyl-Tn antigen (a related mucin-type cancer-associated determinant) was also found in the somatic and deoxycolate extracts, although it was not detected in E/S preparations. Immunohistochemical analysis revealed that Tn is mainly expressed in testis while sialyl-Tn glycoproteins were shown to be more widely distributed, being present in parenchyma, basal membrane of the tegument and in apical membrane of epithelial cells present in the digestive tube. These results are in agreement with those found by Berasain et al. (unpublished data) who found exposed GalNAc in testes and sialic acid in the gut by histochemical analysis using different lectins.

On the basis of their electrophoretic mobility, Tn glycoproteins from F. hepatica were resolved as six components of 10, 37, 76, 125, 170 and 205 kDa, and sialyl-Tn components showed an apparent molecular mass of 28, 32 kDa, and two broad bands of 90-110 and 170-190 kDa. Both antigens seem to be expressed on different glycoproteins, as supported by the results of co-expression analysis of these antigens. This fact agrees with the observation that Tn and sialyl-Tn antigens are not co-expressed in O-glycoproteins of E. granulosus extracts (Alvarez Errico et al., 2001). Considering that Tn is the immediate precursor of sialyl-Tn determinant, we hypothesise that in F. hepatica, all Tn residues of a glycoprotein are good substrates for sialyl-Tn biosynthesis, catalysed by the activity of a sialyltransferase $(\alpha 2, 6$ -sialyltransferase in mammalian cells). In contrast, the T antigen (Gal1,3-GalNAc- α Ser/Thr) was present in glycoproteins expressing Tn, suggesting that not all Tn structures are good substrates for the β -3-Gal-T in *F. hepatica*. This observation could be explained by the fact that the specificity of this enzyme is controlled by the amino acid sequence

of the peptide substrates (Granovsky et al., 1994). Purified Tn-glycoproteins from deoxycolate extract contained other sugars like GlcNAc and/or sialic acid. A fraction of Tn-glycoproteins were soluble in perchloric acid, suggesting that they are highly glycosylated as shown for Tn glycoproteins obtained from human breast carcinoma cells (Pancino et al., 1991; Osinaga et al., 1994). The observation that only the 76 kDa Tn-glycoprotein remained in the 0.6 N perchloric acid-soluble fraction suggests that it could be a good candidate for mucin characterisation in this parasite. On the contrary, the glycosylation pattern of sialyl-Tn-glycoproteins seemed to be different because almost all precipitated under perchloric acid treatment.

Truncated variants of O-glycan chains are good potential targets for immunotherapy (Toyokuni et al., 1994; Danishefsky and Allen, 2000). Although Tn is a glycan structure, it has been reported that some Tn-glycosylated peptides may be capable of binding to proteins of the major histocompatibility complex and inducing T-cell lymphocyte activation (Galli-Stampino et al., 1997). It was shown in animal models that Tn is capable of inducing an effective cellular immune response against Tn-expressing tumour cells, using both natural (Singhal et al., 1991) and synthetic Tn immunogens (Lo-Man et al., 2001). In addition, patients have been immunised against the sialyl-Tn antigen and preliminary studies indicate that patients who developed high antibody titres in their serum survived longer than patients with lower titres (Livingston and Ragupathi, 1997). GalNAc-O-Ser/Thr represents the biochemical definition of Tn, but a lot of evidence suggests that the antigen identified by the immune system may be a more complex structure. Clusters of Tn residues are an essential part of the determinant recognised by mAb (Nakada et al., 1993; Osinaga et al., 2000) and by the Tn-specific human macrophage C-type lectin (Iida et al., 1999), all of which react poorly or are unreactive with glycopeptides bearing Tn structures that are not organised into clusters. When a synthetic dendrimeric multiple antigenic glycopeptide was used in active specific immunotherapy, the tri-Tn glycotope was much more efficient than the mono-Tn analogue in promoting the survival of tumour-bearing mice (Lo-Man et al., 2001). The clustering effect has also been reported on anti-sialyl-Tn mAb. Reddish et al. (1997) have shown that some antibodies recognise the sialyl-Tn as an isolated structure (mAb 195.3) whereas some others need a cluster for binding (as mAb B72.3, used in this work). Tanaka et al. (1999) found that a cluster composed of four sialyl-Tn antigens is the essential epitopic structure for mAb MLS132. The ability to synthesise monomer or cluster of Tn/sialyl-Tn structures on mucin polypeptide backbones could depend in part on the sequence of O-glycosylation sites of the apomucin, as well as on the repertoire and specificity of glycosyltransferases required for Tn/sialyl-Tn synthesis. The biochemical and functional characterisation of Tn and sialyl-Tn glycoproteins purified from F. hepatica could contribute to elucidating the involvement of these proteins in the parasite biology as well as in eventual induction of the host immune response.

Glycosyltransferases constitute a large group of enzymes that are involved in the biosynthesis of oligosaccharides and polysaccharides (Breton et al., 2001). Until now, only the 3- α -GalNAc-transferase, and 2- and 4- α -L-fucosyltransferases have been identified in F. hepatica (Ben-Ismail et al., 1982a), revealing the ability of this parasite to synthesise A, H, and Lewis human blood group antigens (Ben-Ismail et al., 1982b). In this study, we characterised the activity of ppGaNTase from F. hepatica, which represents a key regulatory factor in defining the repertoire of O-glycans (Marth, 1996; Clausen and Bennett, 1996). Peptides derived from the tandem repeat region of human mucins (MUC2 and MUC6), and from T. cruzi and T. brucei mucins, were highly glycosylated. Fasciola hepatica extract was able to glycosylate threonines and serines, suggesting the existence of different ppGaNTase activities in this parasite, which could belong to the same or to different enzymes. The finding that multiple ppGaNTases are involved in O-glycosylation is important in understanding the complexity of this post-translational modification. We are currently cloning a ppGaNTase from this parasite and looking for novel members of this family. The functional characterisation of individual ppGaNTases, as well as the identification of Oglycosylated peptide sequences in F. hepatica, should provide a basis to understand the regulation of O-glycan biosynthesis and the expression of simple mucin-type antigens by this parasite. This new field in glycoimmunobiology research of F. hepatica may help to identify new drug targets and to develop carbohydrate-based vaccines.

Acknowledgements

The authors want to thank Gabriela Maggioli for contributing to the preparation of parasite extracts. This work was supported in part by Comisión Honoraria de Lucha Contra el Cáncer and Comisión Sectorial de Investigación Científica (CSIC) (Uruguay).

References

- Acosta-Serrano, A., Almeida, I.C., Freitas-Junior, L.H., Yoshida, N., Schenkman, S., 2001. The mucin-like glycoprotein super-family of *Trypanosoma cruzi*: structure and biological roles. Mol. Biochem. Parasitol. 114, 143–50.
- Alvarez Errico, D., Medeiros, A., Miguez, M., Casaravilla, C., Malgor, R., Carmona, C., Nieto, A., Osinaga, E., 2001. *O*-glycosylation in *Echinococcus granulosus*: identification and characterization of the carcinoma-associated Tn antigen. Exp. Parasitol. 98, 100–9.
- Ben-Ismail, R., Mulet-Clamagirand, C., Carme, B., Gentilini, M., 1982a. Biosynthesis of A, H, and Lewis blood group determinants in *Fasciola hepatica*. J. Parasitol. 68, 402–7.
- Ben-Ismail, R., Carme, B., Mogahed, A., Niel, G., Gentilini, M., 1982b. Antigen sharing between *Fasciola hepatica* and human erythrocytes. Tropenmed. Parasitol. 33, 11–14.

- Breton, C., Mucha, J., Jeanneau, C., 2001. Structural and functional features of glycosyltransferases. Biochimie 83, 713–8.
- Brockhausen, I., 1993. Clinical aspects of glycoprotein biosynthesis. Crit. Rev. Clin. Lab. Sci. 30, 65–151.
- Brockhausen, I., 1999. Pathways of O-glycan biosynthesis in cancer cells. Biochim. Biophys. Acta 1473, 67–95.
- Clausen, H., Bennett, E., 1996. A family of UDP-GalNAc:polypeptide Nacetylgalactosaminyl-transferases control the initiation of mucin-type O-linked glycosylation. Glycobiology 6, 635–46.
- Colcher, D., Hand, P., Nuti, M., Schlom, J., 1981. A spectrum of monoclonal antibodies reactive with human mammary tumor cells. Proc. Natl. Acad. Sci. USA 78, 3199–206.
- Cummings, R.D., Nyame, D., 1999. Schistosome glycoconjugates. Biochim. Biophys. Acta 1455, 363–74.
- Dalton, J.P., Heffernan, M., 1989. Thiol proteases released in vitro by *Fasciola hepatica*. Mol. Biochem. Parasitol. 35, 161–6.
- Damian, R.T., 1997. Parasite immune evasion and exploitation: reflections and projections. Parasitology 115, S169–75.
- Danishefsky, S.J., Allen, J.R., 2000. From the laboratory to the clinic: a retrospective on fully synthetic carbohydrate-based anticancer vaccines. Angew. Chem. Int. Ed. Engl. 39, 836–63.
- Desseyn, J.L., Guyonnet-Dupérat, V., Porchet, N., Aubert, J.P., Laine, A., 1997. Human mucin gene MUC5B, the 10.7-kb large central exon encodes various alternate subdomains resulting in a super-repeat. Structural evidence for a 11p15.5 gene family. J. Biol. Chem. 272, 3168–78.
- Ellis, L.A., Reason, A.J., Morris, H.R., Dell, A., Iglesias, R., Ubeira, F.M., Appleton, J.A., 1994. Glycans as targets for monoclonal antibodies that protect rats against *Trichinella spiralis*. Glycobiology 4, 585–92.
- Galli-Stampino, L., Meinjohanns, E., Frische, K., Meldal, M., Jensen, T., Werdelin, O., Mouritsen, S., 1997. T-cell recognition of tumor-associated carbohydrates: the nature of the glycan moiety plays a decisive role in determining glycopeptide immunogenicity. Cancer Res. 57, 3214–22.
- Gendler, S.J., Lancaster, C.A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E.N., Wilson, D., 1990. Molecular cloning and expression of human tumor-associated polymorphic epithelial mucin. J. Biol. Chem. 265, 15286–93.
- Granovsky, M., Bielfeldt, T., Peters, S., Paulsen, H., Meldal, M., Brockhausen, J., Brockhausen, I., 1994. UDPgalactose:glycoprotein-N-acetyl-D-galactosamine 3-β-D-galactosyltransferase activity synthesizing O-glycan core 1 is controlled by the amino acid sequence and glycosylation of glycopeptide substrates. Eur. J. Biochem. 221, 1039–46.
- Gum Jr., J.R., Hicks, J.W., Toribara, N.W., Siddiki, B., Kim, Y.S., 1994. Molecular cloning of human intestinal mucin (MUC2) cDNA. Identification of the amino terminus and overall sequence similarity to prepro-von Willebrand factor. J. Biol. Chem. 269, 2440–6.
- Hagen, F., Nehrke, K., 1998. cDNA cloning and expression of a family of UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase sequence homologs from *Caenorhabditis elegans*. J. Biol. Chem. 273, 8268–77.
- Hakomori, S., 1989. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. Adv. Cancer Res. 52, 257–331.
- Hanisch, F.G., 2001. O-glycosylation of the mucin type. Biol. Chem. 382, 143–9.
- Hanna, R.E.B., 1980. Fasciola hepatica: glycocalyx replacement in the juvenile as a possible mechanism for protection against host immunity. Exp. Parasitol. 50, 103–14.
- Ho, S.B., Roberton, A.M., Shekels, L.L., Lyftogt, C.T., Niehans, G.A., Toribara, N.W., 1995. Expression cloning of gastric mucin complementary DNA and localization of mucin gene expression. Gastroenterology 109, 735–47.
- Hounsell, E.F., Davies, M.J., Renouf, D.V., 1996. O-linked protein glycosylation structure and function. Glycoconjugate J. 13, 19–26.
- Iida, S., Yamamoto, K., Irimura, T., 1999. Interaction of human macrophage C-type lectin with O-linked N-acetylgalactosamine residues on mucin glycopeptides. J. Biol. Chem. 274, 10697–705.

- Jentoft, N., 1990. Why are proteins *O*-glycosylated? Trends Biochem. Sci. 15, 291–4.
- Kjeldsen, T., Clausen, H., Hirohashi, S., Ogawa, T., Iijima, H., Hakomori, S., 1988. Preparation and characterization of monoclonal antibodies directed to the tumor-associated O-linked sialosyl-2-6-alpha-N-acetylgalactosaminyl (sialosyl-Tn) epitope. Cancer Res. 48, 2214–20.
- Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 227, 680–5.
- Livingston, P., Ragupathi, G., 1997. Carbohydrate vaccine that induces antibodies against cancer. II. Previous experience and future plans. Cancer Immunol. Immunother. 45, 10–19.
- Lo-Man, R., Vichier-Guerre, S., Bay, S., Deriaud, E., Cantacuzene, D., Leclerc, C., 2001. Anti-tumor immunity provided by a synthetic multiple antigenic glycopeptide displaying a tri-Tn glycotope. J. Immunol. 166, 2849–54.
- Marth, J.D., 1996. Complexity in O-linked oligosaccharide biosynthesis engendered by multiple polypeptide-N-acetylgalactosaminyltransferases. Glycobiology 6, 701–5.
- Mendocino, J., Sangadala, S., 1998. Purification and characterization of UDP-GalNAc:polypeptide N-acetylgalactosaminyl transferase from swine trachea epithelium. Mol. Cell. Biochem. 85, 135–45.
- Menrath, M., Michel, A., Kunz, W., 1995. A female-specific cDNA sequence of Schistosoma mansoni encoding a mucin-like protein that is expressed in the epithelial cells of the reproductive duct. Parasitology 111, 477–83.
- Nakada, H., Inoue, M., Numata, Y., Tanaka, N., Funakoshi, I., Fukui, S., Mellors, A., Yamashina, I., 1993. Epitopic structure of Tn glycophorin A for an anti-Tn antibody (MLS 128). Proc. Natl. Acad. Sci. USA 90, 2495–9.
- Nolan, D.P., Jackson, D.G., Windle, H.J., Pays, A., Geuskens, M., Michel, A., Voorheis, H.P., Pays, E., 1997. Characterization of a novel, stagespecific, invariant surface protein in *Trypanosoma brucei* containing an internal serine-rich, repetitive motif. J. Biol. Chem. 272, 29212– 21.
- Nyame, K., Cummings, R.D., Damian, R.T., 1987. Schistosoma mansoni synthesizes glycoproteins containing terminal O-linked N-acetylglucosamine residues. J. Biol. Chem. 262, 7990–5.
- Osinaga, E., Pancino, G., Porchet, N., Berois, N., De Cremoux, P., Mistro, D., Aubert, J.P., Calvo, F., Roseto, A., 1994. Analysis of a heterogeneous group of human breast carcinoma associated glycoproteins bearing the Tn determinant. Breast Cancer Res. Treat. 32, 139–52.
- Osinaga, E., Babino, A., Grosclaude, J., Cairoli, E., Batthyany, C., Bianchi, S., Signorelli, S., Varangot, M., Musé, I., Roseto, A., 1996. Development of an immuno-lectin-enzymatic assay for the detection of serum carcinoma-associate glycoproteins bearing Tn determinant. Int. J. Oncol. 8, 401–6.
- Osinaga, E., Bay, S., Tello, D., Babino, A., Pritsch, O., Assemat, K., Cantacuzene, D., Nakada, H., Alzari, P., 2000. Analysis of the fine specificity of Tn-binding proteins using synthetic glycopeptide epitopes and a biosensor based on surface plasmon resonance spectroscopy. FEBS Lett. 469, 24–28.
- Pancino, G., Osinaga, E., Vorauher, W., Kakouche, A., Mistro, D., Charpin, C., Roseto, A., 1990. Production of a monoclonal antibody as immunohistochemical marker on paraffin embedded tissues using a new immunization method. Hybridoma 9, 389–95.
- Pancino, G., Osinaga, E., Charpin, C., Mistro, D., Barque, J.P., Roseto, A., 1991. Purification and characterization of a breast-cancer-associated glycoprotein not expressed in normal breast and identified by monoclonal antibody 83D4. Br. J. Cancer 63, 390–8.
- Persat, F., Vincent, C., Schmitt, D., Mojon, M., 1996. Inhibition of human peripheral blood mononuclear cell proliferative response by glycosphyngolipids from metacestode of *Echinococcus multilocularis*. Infect. Immun. 64, 3682–7.
- Reddish, M., Jackson, L., Koganty, R., Qiu, D., Hong, W., Longenecker, B.M., 1997. Specificities of anti-sialyl-Tn and anti-Tn monoclonal antibodies generated using novel clustered synthetic glycopeptide epitopes. Glycoconjugate J. 14, 549–60.

- Singhal, A., Fohn, M., Hakomori, S., 1991. Induction of N-acetylgalactosamine-O-serine/threonine (Tn) antigen-mediated cellular immune response for active immunotherapy in mice. Cancer Res. 51, 1406– 11.
- Tanaka, N., Nakada, H., Inoue, M., Yamashina, I., 1999. Binding characteristics of an anti-Siaalpha2-6GalNAcalpha-Ser/Thr (sialyl Tn) monoclonal antibody (MLS 132). Eur. J. Biochem. 263, 27–31.
- Tettamanti, G., Pigman, W., 1968. Purification and characterization of bovine and ovine submaxillary mucins. Arch. Biochem. Biophys. 124, 41–50.

Towbin, H., Stabelin, T., Gordon, J., 1979. Electrophoretic transfer of

protein from polyacrylamide gels to nitrocellulose sheets. Proc. Natl. Acad. Sci. USA 76, 4350-4.

- Toyokuni, T., Hakomori, S., Singhal, A.K., 1994. Synthetic carbohydrate vaccines: synthesis and immunogenicity of Tn antigen conjugates. Bioorg. Med. Chem. 2, 1119–32.
- Van den Steen, P., Rudd, P., Dwek, R., Opdenakker, G., 1998. Concepts and principles of O-linked glycosylation. Crit. Rev. Biochem. Mol. Biol. 33, 151–208.
- Yoshida, A., Suzuki, M., Ikenaga, H., Takeuchi, M., 1997. Discovery of the shortest sequence motif for high level mucin-type O-glycosylation. J. Biol. Chem. 272, 16884–8.