The variant-specific surface protein of *Giardia*, VSP4A1, is a glycosylated and palmitoylated protein

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The variant-specific surface proteins (VSPs) of the ancient protist *Giardia duodenalis* (syn.: *Giardia intestinalis*, *Giardia lamblia*) are cysteine- and threonine-rich polypeptides that can vary considerably in sequence and size. In the present study, we have purified a VSP (VSP4A1, formerly called CRISP-90) from a cloned *Giardia* isolate, derived from a sheep, by Triton X-114 phase partitioning and anion-exchange chromatography. Analysis of the purified VSP4A1 showed that this protein is post-translationally modified with both glycans and lipid. The glycans of VSP4A1 were detected and partially characterized by (1) compositional analysis, which indicated the presence of GlcNAc and Glc (0.5 and 1.0 mol/mol of protein respectively), and (2) the specific labelling of VSP4A1 with galactosyltransferase/UDP-[³H]Gal. The glycans were released by β -elimination, suggesting that they are O-linked to the protein. Bio-Gel P4

chromatography of the released galactosylated glycans and further compositional analysis suggested that the major glycan on the VSP is a trisaccharide with Glc at the reducing terminus. These and other results indicate the absence of any N-linked glycans on the VSP and suggest instead that it is elaborated with a novel type of short O-linked glycan. Compositional analysis and radiolabelling experiments also indicated that VSP4A1 is modified with covalently linked palmitate (1 mol/mol of protein). Hydroxylamine treatment at neutral pH of [³H]palmitate-labelled VSP4A1 indicated that the acyl chain may be attached by a thioester linkage. A likely location for the lipid modification appears to be in the region of the C-terminal domain where it may facilitate association of the protein with the plasma membrane.

INTRODUCTION

Protozoan parasites of the genus *Giardia* belong to the diplomonad flagellates which most likely represent one of the earliest branches of eukaryotic evolution. *Giardia duodenalis*-type organisms (syn.: *Giardia lamblia*, *Giardia intestinalis*) colonize the small intestine of a wide variety of different vertebrate hosts and are the causative agents of the most common protozoal intestinal infection of humans world wide [1]. Analyses of *Giardia* using a number of complementary techniques have demonstrated that the greatest degree of diversity among isolates of this organism resides in their major surface antigens. These comprise a group of novel polypeptides that are called variant-specific surface proteins (VSPs) and are components of a coat covering the entire cell surface of the parasite [2–4]. Although these polypeptides can vary considerably in both sequence and size, they share several common structural features [5–8]. These include a high content of cysteine (approx. 12 mol%) and threonine (approx. 11 mol%), the presence of multiple copies of the tetrapeptide CXXC motif and a highly conserved C-terminal sequence. The biological function of these proteins is still unclear, but is believed to be associated with the survival of the parasite in the vertebrate small intestine.

Previous studies in our laboratory have shown that a cloned isolate of *Giardia* obtained from a sheep (clone O2-4A1) expresses a VSP (VSP4A1, formerly called CRISP-90) that migrated under

reducing SDS/PAGE conditions at 90 kDa [9]. The ability of the O2-4A1 clone to undergo variation of its VSP was shown by the spontaneous appearance of new cysteine-rich proteins in clonally derived populations during prolonged *in vitro* culture and in cultures that had survived the treatment with a cytotoxic anti-VSP4A1 serum. In the current study, we describe the purification of VSP4A1 from trophozoites of the variant clone O2-4A1 and demonstrate that it belongs to a novel class of palmitoylated and O-linked GlcNAc-bearing surface glycoproteins.

MATERIALS AND METHODS

Organisms and culture

G. duodenalis clone O2-4A1 which predominantly expresses VSP4A1 was used throughout this study [9]. Clone O2-4A1 is a subline of the CH-O2 isolate originally derived from a sheep in Switzerland [10]. Trophozoites were cultivated and harvested as previously described [9].

Purification of VSP4A1

Cells [(2–3) × 10⁹] were resuspended in 6 ml of 20 mM Tris/HCl/150 mM NaCl, pH 7.5 (TBS) containing 2% Triton X-114 (Boehringer-Mannheim) and the proteinase inhibitors aprotinin

Abbreviations used: GC/MS, gas chromatography–mass spectrometry; GU, glucose unit; HPAEC, high-pH anion-exchange chromatography; HPTLC, high-performance thin-layer chromatography; O-GlcNAc, O-linked GlcNAc; pI, isoelectric point; LDAO, *N,N*-dimethyldodecylamine *N*-oxide; TBS, Tris-buffered saline; TMS, trimethylsilyl; VSP, variant-specific surface protein.

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(10 µg/ml), leupeptin (2 µg/ml) and pepstatin (2 µg/ml) and 2 mM EDTA. The lysed cell suspension was mixed and allowed to stand on ice for 20 min with occasional shaking in between. The lysate was centrifuged for 5 min at 4000 *g* and 4 °C to remove cytoskeletal components. Subsequently, the supernatant was subjected to phase partition with Triton X-114 using a modification of the method originally described by Bordier [11]. After the addition of Bromophenol Blue (Bio-Rad) to a final concentration of 0.001 %, the sample was mixed and placed on ice for 10 min followed by incubation for 10 min at 37 °C. This treatment resulted in the formation of a detergent-enriched and a detergent-depleted phase (aqueous phase). This solution was centrifuged for 5 min at 2500 *g* and room temperature. The top aqueous phase was retained and re-extracted with an additional amount of Triton X-114 in TBS to yield a final detergent concentration of 1 %. The solution was mixed, left on ice for 10 min and finally overlaid on the detergent phase obtained from the first separation step. Incubation at 37 °C and centrifugation were performed as described above. The top aqueous phase was discarded and the detergent phase was washed twice with 4 vol. of 0.05 % Triton X-114 in TBS followed by incubation and centrifugation as described above. The washed detergent phase was stored at -20 °C or immediately used for further purification.

The fraction obtained from the Triton X-114 phase partition was diluted with 4 vol. of TBS and mixed thoroughly. Subsequently, the solution was diluted another four times with a degassed solution containing 2.75 mM *N,N*-dimethyldodecylamine *N*-oxide (LDAO) in 20 mM Tris/HCl, pH 7.5. The solution was placed on ice for 20 min with occasional shaking and then applied to a DEAE-cellulose C-545 (Pharmacia) column (0.8 cm × 3.5 cm) equilibrated with de-gassed 20 mM Tris/HCl, pH 7.5, containing 30 mM NaCl and 2.2 mM LDAO. The column was then washed at room temperature with 10 ml of the equilibration buffer followed by 10 ml portions of increasing NaCl concentration (0.05, 0.09, 0.15 and 1 M) in a degassed solution of 20 mM Tris/HCl/2.2 mM LDAO, pH 7.5. Fractions of 10 ml were collected and those containing VSP4A1, which appeared at 0.15 M NaCl, were pooled and concentrated by centrifugation at 2500 *g* and 4 °C in cellulose or, in the case of carbohydrate analysis, in polysulfone ultrafiltration units with a 30 kDa cut-off (Millipore). The concentrated samples were washed twice in the same ultrafiltration units with 2 ml of 10 mM Tris/HCl/10 mM NaCl, pH 7.5, and finally resuspended in 250 µl of this buffer or water. The purity of VSP4A1 was analysed by SDS/PAGE.

Protein quantification and SDS/PAGE

Protein was determined by the Bio-Rad protein assay based on the method of Bradford [12]. Purified VSP4A1 was determined by quantitative amino acid analysis using an ABI 420 H/A (Applied Biosystems) or Aminoquant (Hewlett-Packard) analyser. SDS/PAGE and fluorography were performed as described by Bruderer et al. [9].

Determination of the isoelectric point (pI)

The pI of VSP4A1 was determined by isoelectric focusing using the 2117 Multiphor Electrophoresis Unit in conjunction with Ampholine PAG plates (pH range 3.5–9.5) (LKB Bromma/Pharmacia). The broad pI markers were from Pharmacia. The sample analysed was a DEAE-cellulose-purified VSP4A1 fraction derived from a *Giardia* culture metabolically labelled with [³⁵S]cysteine and containing 10 µg of protein diluted in 15–20 µl of water. In additional experiments, samples were denatured in

the presence of 75 mM dithiothreitol either in 8 M urea or by boiling for 5 min. Electrofocusing, fixation and staining of the gel were performed as described by the manufacturers.

Biosynthetic labelling with radioactive monosaccharides and [³H]palmitate

Trophozoites [(1–15) × 10⁷] were labelled with 50–400 µCi of the following monosaccharides: D-[6-³H]Gal (40 Ci/mmol; Amersham), D-[6-³H]Glc (36.2 Ci/mmol; NEN), D-[2-³H]Man (22.7 Ci/mmol; NEN) and D-[6-³H]GlcN (38.8 Ci/mmol; NEN). The labelling was carried out at 37 °C in 0.5 or 1 ml of RPMI 1640 medium (Gibco) without glucose during a 3 h incubation that followed a 30 min preincubation period in the absence of the radioactive monosaccharide. For biosynthetic labelling with radioactive palmitate, trophozoites (1 × 10⁸) were incubated for 1.5 h at 37 °C in 1 ml of RPMI 1640 containing 1 mCi of [9,10-³H]palmitic acid. Before incubation, 1 mCi (30–60 Ci/mmol; NEN) of radiolabelled palmitate stock solution was dried under a nitrogen stream, resuspended in 10 µl of 100 % ethanol and mixed with 6 µl of 100 mg/ml fatty acid-free BSA (a gift from Dr. A. Dieckmann-Schuppert, Center for Hygiene and Medical Microbiology, University of Marburg, Germany) in RPMI 1640. After 1 h of shaking at room temperature, the palmitate-BSA complex was added to the cell suspension and labelling was initiated. For analysis by SDS/PAGE and fluorography either cell lysates or purified VSP4A1 or VSP4A1 precipitated from these lysates with anti-VSP4A1 serum were used following the methods described elsewhere in this paper and by Bruderer et al. [9].

Demonstration of carbohydrate by a glycan-detection kit

In an initial attempt to detect possible carbohydrate moieties in VSP4A1 a commercial glycan-detection kit (DIG glycan detection kit; Boehringer-Mannheim) was applied. Glycan detection was achieved after blotting of the VSP from an SDS/polyacrylamide gel to a nitrocellulose filter and treatment with the glycan-detection kit reagents as described by the manufacturer. In addition to VSP4A1 (4 µg, alkylated or reduced form), the control glycoprotein transferrin (4 µg) and the non-glycosylated protein creatinase (4 µg) were also included in the test.

Treatment with *N*-glycosidase

The enzyme used was recombinant *N*-glycosidase F from *Flavobacterium meningosepticum* (25000 units/mg; Boehringer-Mannheim). VSP (5 µg) was denatured by boiling for 2 min in 10 µl of 1 % (w/v) SDS solution containing 1 % β-mercaptoethanol and protease inhibitors at the concentrations given above. Subsequently, the sample received 50 µl of a solution consisting of 90 mM sodium phosphate, 1 % (v/v) Triton X-100, pH 7.3, and water to a final volume of 95 µl. The sample was boiled for another 2 min and left to cool down. Finally, 0.8 unit of the enzyme and another portion of protease inhibitors were added. The 100 µl mixture was incubated for 18 h at 37 °C. Transferrin (Boehringer-Mannheim) was employed as the positive control. After the incubation, the mixture was boiled for 5 min to inactivate the enzyme, and the proteins were precipitated with methanol/chloroform as described below and subjected to SDS/PAGE.

Radiolabelling of VSP4A1 with bovine milk galactosyltransferase

Purified VSP4A1 was labelled using bovine milk 4β-galactosyltransferase and UDP-[³H]Gal as described previously [13]. In

brief, bovine milk galactosyltransferase was autogalactosylated in the presence of UDP-Gal and extensively dialysed before use. Purified VSP4A1 (2–20 µg of protein) was suspended in 50 mM Hepes/NaOH, pH 7.3, containing 5 mM MnCl₂, 1 mM β-mercaptoethanol and 5 µCi of UDP-[4,5-³H]Gal (10–25 mCi/mmol; NEN) and galactosyltransferase (0.4 units) in a final volume of 50 µl and incubated at 37 °C for 1 h. The labelled protein was separated from unincorporated UDP-[³H]Gal by solvent precipitation as described by Wessel and Flügge [14], and resuspended in water. A number of ³H-labelled galactosylated disaccharide and trisaccharide standards were also synthesized under identical conditions using GlcNAc, Glc, GlcN and chitobiose (GlcNAcβ1-4GlcNAc) as substrates. After incubation with galactosyltransferase and UDP-[³H]Gal, the labelled products were desalted by passage down a column of AG 50 (X12; H⁺ form) over AG 3 (X8; OH⁻ form) (Bio-Rad), except for Galβ1-4GlcN, which was desalted on Dowex 1 (X8) (Bio-Rad). The level of incorporation into chitobiose, Glc and GlcN relative to GlcNAc (100 %) was 91, 31 and 8 % respectively. The amount of label incorporated into 10 µg (0.15 nmol) of VSP was equivalent to that incorporated into 0.5 nmol of GlcNAc.

Labelled VSP4A1 was subjected to the following treatments to confirm the specificity of labelling. Proteinase K digestions were carried out in 0.1 M Hepes/NaOH, pH 7.4, containing 2 mM CaCl₂ (2 units; 37 °C; 18 h) and the products analysed on a column (1 cm × 50 cm) of Sephadex G-25 eluted in 0.1 M ammonium acetate. Immunoprecipitation was carried out as previously described by Bruderer et al. [9], with some modifications. The precipitation procedure was repeated three times and the immunocomplexes were suspended and combined in electrophoresis buffer. Samples were heated at 95 °C for 10 min and analysed by SDS/PAGE. Labelled VSP was detected on the gels by fluorography as described above. Labelled VSP was also treated with bovine testicular β-galactosidase (Boehringer-Mannheim) or strong trifluoroacetic acid to confirm the addition of βGal residues. β-Galactosidase digestion was carried out in 0.1 M sodium citrate/sodium phosphate, pH 4.5 (35 m-units; 37 °C; 18 h). The reaction was stopped by boiling for 5 min and samples were desalted by passage down a column of AG 50 (X12; H⁺ form) over AG 3 (X8; OH⁻ form). Acid hydrolysis was carried out with 2 M trifluoroacetic acid (100 °C; 2 h); the acid was removed under vacuum. The released monosaccharides were analysed by high-pH anion-exchange chromatography (HPAEC) as described below.

Release of labelled glycans and palmitic acid

The release of [³H]Gal-labelled glycans from VSP4A1 was monitored after β-elimination, HNO₂ deamination or mild acid hydrolysis. β-Elimination was carried out in 0.1 M NaOH containing 0.5 M NaBH₄ (100 ml) at 37 °C for 18 h. Samples were acidified with 0.1 M acetic acid, dried under nitrogen and borate salts were removed by coevaporation with methanol (5 × 250 µl). The reduced glycans released by this procedure were directly analysed by Bio-Gel P4 chromatography or desalted on a column of AG 50 (X12; H⁺ form) over AG 3 (X8; OH⁻ form) before analysis by HPAEC. HNO₂ deamination and reduction was carried out as described previously [15]. Mild acid hydrolysis was carried out using conditions (1 M HCl; 100 °C; 6 min) used to hydrolyse GlcNAc-1-PO₄ linkages [16].

To determine the nature of the fatty acid–protein linkage, VSP4A1 (5–10 µg) purified from trophozoites metabolically labelled with [9,10-³H]palmitic acid were dissolved in 80 µl of either 1 M hydroxylamine/HCl, pH 7.5, or 10 mM Tris/HCl, pH 7.5 and incubated for 2 h at room temperature. The samples

were washed by ultrafiltration, and the VSP was analysed for the presence or absence of radiolabelled palmitate by SDS/PAGE and fluorography as described above. Additional experiments were performed to confirm that the fluorographic signals detected were due to covalent attachment of tritiated palmitate to VSP. For this purpose, [³H]palmitate-labelled trophozoites (1 × 10⁸) were suspended in 140 µl of TBS containing 0.8 % Triton X-100 and proteinase inhibitors (see above). After sonication on ice for 10 s at 20 W (Branson Sonifier 250), the cell lysate was centrifuged (4000 g; 5 min) and the supernatant vacuum dried. Non-covalently bound lipids were extracted from the dried material with chloroform/methanol as described by Bouvier et al. [17]. The final precipitate was then subjected to SDS/PAGE and fluorography.

HPAEC and Bio-Gel P4 chromatography

HPAEC was performed on a Dionex model BioLC HPLC column, equipped with a pulsed amperometric detector. Labelled monosaccharides and reduced glycans were fractionated on a CarboPac PA1 column (4 mm × 250 mm) Dionex Corp.) in 15 mM NaOH at a flow rate of 0.6 ml/min. Monosaccharides were identified by their coelution with co-injected monosaccharide standards. The elution times of the β-eliminated glycans were compared with a series of co-injected reduced mono- and oligo-saccharides. High-resolution gel filtration was carried out on a column (1.5 cm × 100 cm) of Bio-Gel P4 (400 mesh) (Bio-Rad) eluted in either water or 0.1 M ammonium acetate at 0.2 ml/min. Labelled glycans were detected by scintillation counting and their elution times compared with a series of dextran oligosaccharides (prepared by partial acid hydrolysis of dextran) which were co-injected with the sample and detected with either a refractive index monitor (Erma Inc.) or by spotting an aliquot of each fraction on a high-performance thin-layer chromatography (HPTLC) silica-gel 60 sheet (Merck) and detecting carbohydrate with the orcinol/H₂SO₄ reagent. For analysis of the glycans on silica-gel 60 HPTLC, the sheets were developed in butan-1-ol/acetone/water (9:6:5, by vol.) [18], then sprayed with En³Hance (NEN) for detection of labelled glycans by fluorography.

Monosaccharide and lipid analysis

For monosaccharide and lipid analysis, purified VSP4A1 (50 µg) was subjected to solvolysis in 0.5 M methanolic HCl (50 µl; 80 °C; 15 h). In some experiments, glycans were first released by β-elimination and desalted on a small column of mixed-bed ion-exchange material before solvolysis. Samples were neutralized and amino sugars N-acetylated by the addition of 10 µl of pyridine followed by 10 µl of acetic anhydride (25 °C; 20 min). The solvents were then removed under vacuum and the released monosaccharides derivatized with chlorotrimethylsilane/hexamethyldisilazane/pyridine (1:3:9, by vol.). Combined gas chromatography–mass spectrometry (GC/MS) was performed on a Hewlett–Packard 5980-MSD system using a capillary HP-1 column (Hewlett–Packard) or a capillary SE 54 fused-silica column (0.25 mm × 30 m) (Alltech Associates) as described previously [15].

RESULTS

Purification of VSP4A1

VSP4A1 was extracted from *Giardia* trophozoites with Triton X-114 and, after phase partitioning, was quantitatively recovered in the detergent-enriched phase (Figure 1A, lane 3). When this

Figure 1 Electrophoretic analysis of VSP4A1 in the various purification stages

(A) Coomassie-stained SDS/polyacrylamide gel representing fractions of the Triton X-114 phase separation. Lane 1, total cell lysate; lane 2, supernatant of the 4000 *g* centrifugation step; lane 3, detergent phase of the Triton X-114 phase separation. Samples loaded on to the gel represent the same percentage of the fraction's volume. (B) SDS/polyacrylamide gel with the VSP4A1-containing fraction from the DEAE-cellulose chromatography that follows the Triton X-114 phase separation.

phase was subjected to DEAE-cellulose chromatography, the VSP was eluted at 150 mM NaCl as a pure protein, as determined by SDS/PAGE analysis (Figure 1B). The phase-separation step resulted in a 14-fold purification of the protein with negligible loss. After DEAE-cellulose chromatography and ultrafiltration, the VSP was recovered with a yield of approx. 60% and was essentially homogeneous when analysed by SDS/PAGE as detected by Coomassie Blue staining. The pI of the folded protein was determined to be 6.1. However, when isoelectric focusing was performed after samples had been boiled or incubated in 8 M urea, in the presence of dithiothreitol, four distinct protein bands with pI values between 4.5 and 5.0 were observed (results not shown). One of these bands probably corresponds to the fully denatured reduced molecule, and the others may represent partially reoxidized VSP4A1 conformations.

The 90 kDa apparent molecular mass of the protein, as estimated for the reduced form by SDS/PAGE, is considerably larger than that predicted from the corresponding gene sequence for the mature protein (Ph. Papanastasiou, unpublished work), implying that VSP4A1 may be post-translationally modified. Subsequent analysis revealed the presence of two distinct types of post-translational modification.

Evidence for glycosylation of VSP4A1

The presence of some form of glycosylation was suggested after probing the nitrocellulose-immobilized VSP with the DIG glycan-

Figure 2 Identification of the VSP4A1 as a glycoprotein using the DIG glycan detection kit

The SDS/polyacrylamide gel was blotted on to nitrocellulose and processed for the presence of glycoprotein as described in the Experimental section. Lane 1, VSP4A1; lane 2, transferrin (positive control); lane 3, creatinase (negative control).

detection kit. The procedure utilizes a periodate oxidation step to generate aldehyde groups in the glycan moiety which are subsequently linked to digoxigenin, allowing the sensitive detection of a broad range of glycan structures. As shown in Figure 2, both VSP4A1 (lane 1) and the glycoprotein standard, transferrin (lane 2), were strongly recognized by a digoxigenin-specific antibody, whereas the non-glycosylated protein, creatinase (lane 3), was not. Monosaccharide analysis of the purified protein revealed the presence of approx. 0.5 mol of GlcNAc and 1 mol of Glc per mol of VSP (Figure 3, Table 1). The levels of Glc were at least 3-fold higher than in a blank sample taken through the same purification scheme (GlcNAc was not detected at all in these samples) and were present after microdialysis or solvent precipitation of the VSP. Although Glc is a common contaminant in monosaccharide analysis, these results suggest that Glc may be a constituent of the VSP4A1 glycans.

The above data suggest that VSP4A1 contained terminal GlcNAc and/or Glc residues which should be labelled by incubating the protein with bovine milk galactosyltransferase and UDP-[^3H]Gal [13]. As shown in Figure 4(A), VSP4A1 was labelled by this procedure, with the amount of label being proportional to the amount of added protein. Labelling was specific for the VSP on the basis of several criteria. First, the labelled product was susceptible to proteinase K digestion. Before digestion, all the label was eluted in the void volume of a Sephadex G-50 column, whereas after proteolysis most of the label was eluted with the buffer salts in the total volume (results not shown). Secondly, the labelled protein was quantitatively immunoprecipitated with anti-VSP4A1 serum. When analysed by SDS/PAGE, the immunoprecipitated protein had a slightly higher apparent molecular mass than the native VSP, indicating that the addition of Gal may affect its electrophoretic mobility (Figure 4B, lane 1). Thirdly, analysis of the labelled monosaccharides released by β -galactosidase digestion or acid hydrolysis indicated that all the incorporated label was present as β -Gal residues as expected (Figure 4C). Attempts to confirm the presence of glycosylation by metabolic labelling were unsuccessful as various ^3H -labelled monosaccharides were either not incorporated into protein (i.e. D-[2- ^3H]Man and D-[6- ^3H]Gal) or incorporated into the protein as non-sugar constituents (i.e. D-[6- ^3H]Glc and D-[6- ^3H]GlcN).

Figure 3 Compositional analysis of VSP4A1

Purified VSP4A1 was subjected to acid methanolysis and the released methyl glycosides and fatty acid methyl esters were analysed by GC/MS as their trimethylsilyl (TMS) derivatives. The profiles show the total ion chromatogram (TIC). Peaks corresponding to Glc (1), palmitic acid (2), *scyllo*-inositol internal standard (3) and GlcNAc (4) are marked. The two Glc peaks correspond to the α and β anomers of glucopyranose. Peaks marked with an asterisk are background contaminants.

Table 1 Monosaccharide analysis of the native VSP4A1 glycoprotein and the glycans released by β -elimination

Native glycoprotein fractions were subjected to methanolysis and the released monosaccharides analysed by GC/MS as their TMS derivatives (expressed as mol/mol of protein). Low levels of Glc were also found when a blank sample was taken through the same purification procedure (Blank). These values have been subtracted from those obtained from the native VSP4A1 analysis. The glycans released by reductive β -elimination were also subjected to monosaccharide analysis before and after methanolysis to detect free and total monosaccharides respectively. MS, monosaccharides. —, not detected.

Monosaccharide	Blank	Native VSP4A1	β -Eliminated glycans	
			Total MS	Free MS
GlcNAc	—	0.416	0.315	—
Glc	0.450	1.078	0.780	—
Glucitol	0.039	—	0.315	0.162

Partial characterization of the VSP4A1 glycan

The absence of mannose in the monosaccharide composition suggested that the glycans were not N-linked. This is consistent with the finding that *N*-glycosidase treatment failed to change the electrophoretic mobility of the native VSP or to release any of the label from the galactosyltransferase-labelled VSP (results not shown). Reductive β -elimination of [3 H]Gal-labelled VSP4A1 released all of the label as low-molecular-mass material (Figure 5) suggesting an O-glycosidic linkage to serine or threonine residues. The β -eliminated glycans retained the same monosaccharide composition as the native protein. After methanolysis, all the GlcNAc was recovered as the TMS derivative of 1-*O*-methyl-*N*-acetylglucopyranoside, suggesting that this residue is not linked to protein at the reducing terminus of the glycan. In contrast, approx. 30 % of the glucose was recovered as the TMS derivative of glucitol, providing evidence that some of the glucose may be linked to protein and be reduced when it is cleaved from

Figure 4 Labelling of VSP4A1 with galactosyltransferase/UDP- ^3H Gal

(A) Various amounts of VSP4A1 were incubated with bovine milk galactosyltransferase and UDP- ^3H Gal and incorporation of counts determined after solvent precipitation of protein. (B) SDS/PAGE analysis of galactosyltransferase/UDP- ^3H Gal-labelled VSP4A1. ^3H Galactosylated VSP4A1 was detected by fluorography after analysis by SDS/PAGE. Lane 1, after *in vitro* galactosylation, the ^3H -labelled protein fraction was immunoprecipitated with the anti-VSP4A1 serum and electrophoretically separated under reducing conditions (galVSP4A1, galactosylated VSP4A1). The arrow below the galVSP4A1 indicates the position of non-galactosylated VSP4A1. Lane 2, half of the labelled fraction that did not react with the antiserum (supernatant of the immunoprecipitation), run under reducing conditions. Lane 3, as lane 2, but run under non-reducing conditions. Molecular mass markers (in kDa) are indicated on the left. (C) Release of ^3H Gal from labelled VSP4A1 with β -galactosidase. Galactosyltransferase/UDP- ^3H Gal-labelled VSP4A1 was incubated with bovine testicular β -galactosidase and the released monosaccharides analysed by HPAEC. The elution positions of co-injected monosaccharide standards are indicated at the top of the panel.

Figure 5 β -Elimination of [^3H]Gal-labelled VSP4A1

Galactosyltransferase/UDP-[^3H]Gal-labelled VSP4A1 was fractionated on a column of Sepharose G-50 before ($\square$) or after ($\circ$) incubation in 0.1 M NaOH/0.5 M NaBH $_4$ (37 °C; 18 h). The void (V_0) and total (V_T) volume of the column are indicated.

Figure 6 Bio-Gel P4 analysis of [^3H]Gal-labelled glycans released by reductive β -elimination

Galactosyltransferase/UDP-[^3H]Gal-labelled VSP4A1 was subjected to reductive β -elimination, and the released glycans were analysed directly on a Bio-Gel P4 column eluted in 0.1 M ammonium acetate, pH 7.0. The elution of co-injected dextran oligosaccharides (1–9), Gal/ β 1-4GlcNAc (A) and Gal/ β 1-4GlcNAc/ β 1-4GlcNAc (B) are indicated. V_0 , void volume.

the protein by β -elimination. Low levels of glucitol were also detected when the glycan fraction was analysed directly without methanolysis. This may indicate the presence of a small amount of free glucose in the original VSP4A1 sample or the substitution of the protein with monomeric Glc which would be released as glucitol after β -elimination. Linkage to the protein via a phosphodiester bond [16] is unlikely as very little label (approx. 10%) was released after mild acid hydrolysis that cleaves either Glc-1-PO $_4$ or GlcNAc-1-PO $_4$ linkages (results not shown). Finally, less than 10% of the ^3H label was released by HNO $_2$ deamination, indicating that the GlcN is present exclusively as GlcNAc and not as GlcN.

When the products of reductive β -elimination were analysed by Bio-Gel P4 chromatography, a major peak at 5.3 glucose units (GU) and a minor peak at 4.6 GU were observed (Figure 6). These components have a larger hydrodynamic volume than

Figure 7 Analysis of [^3H]palmitic acid incorporation into VSP4A1 by SDS/PAGE and release of protein-bound radiolabelled fatty acid with hydroxylamine

(A) SDS/PAGE of VSP4A1 purified from *Giardia* biosynthetically labelled with [^3H]palmitic acid. The radiolabelled VSP4A1 was submitted to electrophoresis in its alkylated form and stained with Coomassie Brilliant Blue. Lane 1, control sample not treated with hydroxylamine; lane 2, sample treated with 1 M hydroxylamine, pH 7.5. (B) Fluorograph of [^3H]palmitate-labelled VSP4A1 separated by SDS/PAGE shown in (A). (C) Fluorograph of an SDS/polyacrylamide gel of [^3H]palmitate-labelled VSP4A1-expressing trophozoites. Protein derived from a cell lysate supernatant centrifuged at 4000 g. Lanes 1 and 2, control samples not extracted with organic solvents and containing proteins in their non-reduced and alkylated forms respectively; lane 3, alkylated sample subjected to organic solvent extraction before electrophoresis. The arrows indicate the position of VSP4A1. Molecular-mass standards are shown on the left.

Gal- β 1-4GlcNAc (3.6 GU), the expected product if VSP4A1 was substituted with single O-GlcNAc residues (Figure 6). The 5.3 GU peak has a unique chromatographic mobility on Bio-Gel P4 when compared with several other GlcNAc-containing oligosaccharides (Figure 6). Taken together with the compositional analysis of the β -eliminated glycan, these data suggest that VSP4A1 is substituted with short di- or tri-saccharides that contain Glc at their reducing terminal.

The remainder of the base-released material was eluted as a broad incompletely resolved peak from around 6 GU up to and including the void volume. This material was eluted exclusively in the void volume of the Bio-Gel P4 column when the column was eluted with distilled water, consistent with it being negatively charged. It also bound strongly to cation-exchange resins (i.e. AG 3) indicating the presence of positive charges (results not shown). Although the nature of these components has not been further characterized, it is possible that it represents a mixture of glycopeptides which are generated by partial degradation of the polypeptide backbone.

Evidence for palmitoylation in VSP4A1

The second modification we detected in VSP4A1 is its palmitoylation. As can be seen in the fluorograph of Figure 7(B, lane 1), after biosynthetic labelling of trophozoites with [³H]palmitic acid, a strongly labelled component was detected corresponding in electrophoretic mobility to the 99 kDa alkylated form of VSP4A1. As also seen in Figure 7(B, lane 2), most of the [³H]palmitate-derived incorporation of radiolabel into the VSP was released after neutral hydroxylamine treatment, consistent with a thioester rather than an O-ester linkage. Evidence that this label was due to the incorporation of palmitic acid into VSP4A1 was provided by GC/MS compositional analysis which confirmed the presence of palmitic acid (approx. 1 mol/mol of protein) as the sole fatty acid component in the purified VSP (Figure 3). Covalent attachment of the tritiated palmitate was confirmed by demonstrating the resistance of the protein-associated radiolabel to organic solvent extraction. Electrophoretic separation of protein obtained from a lysate of trophozoites biosynthetically labelled with [³H]palmitic acid demonstrated that thorough extraction of the protein sample with chloroform/methanol before SDS/PAGE did not diminish the intensity of the VSP signal as compared with untreated control samples but resulted in the removal of most of the radiolabelled free lipids contained in the lysate (Figure 7C). The latter analysis also illustrates the shift in electrophoretic mobility between VSP4A1 samples prepared under non-reducing (lane 1) or alkylating conditions (lanes 2 and 3), a phenomenon previously found to be characteristic of this VSP [9].

DISCUSSION

The trophozoite stage of *Giardia* expresses on its surface one of a series VSPs that are believed to play a central role in the survival of the organism in its natural environment [4,19]. In the present work, we have purified one species of this protein family, VSP4A1, by a combination of detergent-phase partition and anion-exchange chromatography. The purification of a second VSP (VSPrB2) expressed by a progeny of the VSP4A1 variant population that had survived the treatment with a cytotoxic anti-VSP4A1 serum required a slight modification to this methodology, indicating that this procedure may not be applicable as a general purification scheme for the structurally highly diverse group of VSPs (T. Bruderer, unpublished work). Recently, an alternative method for the purification of VSPs based on immunoaffinity chromatography was described by Luján et al. [20]. The pI of VSP4A1, as calculated from the sequence of the corresponding gene using the Gene works software, was found to be 4.5, which is in accordance with the values of around 5.0 predicted for other VSPs [6] but in contrast with the less acidic value of 6.1 experimentally determined by isoelectric focusing. However, when the protein was electrophoresed in its denatured reduced form, a band with a pI equal to that predicted from the primary structure was identified as part of a variety of other distinct bands focusing between pH 4.5 and 5.0. These findings suggest that the pI of 4.5 is only correct for the fully reduced randomly coiled polypeptide chain, whereas the non-reduced and the other reduced but partially refolded conformations formed on thiol group reoxidation have less acidic pI values.

Although the sequences of several VSPs, including VSP4A1, have been determined, little is known about their tertiary structure and the presence of post-translational modifications. Previous studies have failed to detect the presence of carbohydrate in VSPs of human-derived *Giardia* isolates [20,21].

However, in this study we provide strong evidence that at least one of these proteins, VSP4A1, is substituted with a novel form of O-glycosylation. The sensitivity of these glycans to mild base treatment suggests that they may be linked to either serine or threonine residues in the protein. Compositional and high-resolution gel-filtration analyses suggest that these O-linked glycans contain GlcNAc and Glc in short di- or tri-saccharide chains. Glc appears to be present at the reducing terminus of the β -eliminated glycans, while the high efficiency of labelling of these glycans with the bovine milk 4 β -galactosyltransferase suggests that a GlcNAc residue is present at the non-reducing terminus. Although the stoichiometry is consistent with a single glycosylation site per molecule of protein, it is also possible that a subpopulation of VSP4A1 is much more heavily glycosylated.

Considerable variability is found in the O-glycosylation of proteins in the parasitic protozoa. Many parasite proteins are substituted with single O-GlcNAc residues [22–24] which may be further substituted with oligosaccharide chains [25–27]. A distinct type of O-glycosylation is found in *Leishmania* spp., where several secreted proteins have been found to be heavily substituted with elaborate phosphoglycan chains that are linked to serine residues via Man-1-PO₄ linkage [28]. The O-glycans of the *Giardia* VSP4A1 differ in their monosaccharide composition from any of these glycans and are unusual in containing a reducing Glc residue. O-linked Glc has been found in a number of mammalian proteins, although it is usually further substituted with xylose residues [29]. The O-linked glycans of *Giardia* thus appear to be unique. The functional significance of this type of glycosylation remains to be determined. As novel structures, these glycans may contribute to the antigenic variability of the VSPs. In this regard it is important to note that according to our estimations there are approx. 5×10^6 copies of VSP4A1 per cell, making any attached glycans abundant surface constituents. These glycans may also be accessible on the cell surface for mediating specific host–parasite interactions, or be involved in facilitating protein folding and the structural stability of surface coats.

The other modification demonstrated in the present study for a VSP is that VSP4A1 is palmitoylated. The precise location of the covalently attached acyl group is as yet unclear, but other experimental work in our laboratory on the *in vitro* release of the VSP from intact cells has shown that it is within the conserved C-terminal region of the protein [30]. This cleavable segment contains two conserved cysteine residues (Ph. Papanastasiou, unpublished work), one of which may serve as the acylation site. Previous work by other authors failed to detect any fatty acid incorporation into VSP after biosynthetic labelling of trophozoites with tritiated palmitic or myristic acid [31]. This discrepancy cannot be explained at present, but may be due to the different labelling protocols used in the two studies. Fatty acid-modified proteins are of widespread occurrence in eukaryotic cells, but the functional role of protein acylation is not well understood. Long-chain fatty acyl groups are believed to act as membrane anchors for proteins and to play a role in the targeting of their attached proteins as well as in signalling processes [17,32]. Whether palmitoylation of VSP4A1, and possibly other VSPs, may serve to target and anchor its attached protein to the cell membrane or have any other function remains to be determined.

We thank Ms. Cordelia Bommeli for invaluable technical assistance. This work was supported by grants from the Swiss National Science Foundation (31-27645.89) and the Australian National Health and Medical Research Council. M.J.M. is a Wellcome Trust Senior Research Fellow.

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