Isolation and characterization of glycosylphosphatidylinositol-anchored, mucin-like surface glycoproteins from bloodstream forms of the freshwater-fish parasite *Trypanosoma carassii*

Antje LISCHKE*, Christian KLEIN*, York-Dieter STIERHOF*, Michaela HEMPEL*, Angela MEHLERT†, Igor C. ALMEIDA†, Michael A. J. FERGUSON† and Peter OVERATH*¹

*Max-Planck-Institut für Biologie, Abteilung Membranbiochemie, Corrensstrasse 38, D-72076 Tübingen, Germany, and †Department of Biochemistry, The University Dundee, Dundee DD1 5EH, Scotland, U.K.

Wild and farmed freshwater fishes are widely and heavily parasitized by the haemoflagellate *Trypanosoma carassii*. In contrast, common carp, a natural host, can effectively control experimental infections by the production of specific anti-parasite antibodies. In this study we have identified and partially characterized mucin-like glycoproteins which are expressed in high abundance $[(6.0 \pm 1.7) \times 10^6 \text{ molecules} \cdot \text{cell}^{-1}]$ at the surface of the bloodstream trypomastigote stage of the parasite. The polypeptide backbone of these glycoproteins is dominated by threonine, glycine, serine, alanine, valine and proline residues, and is modified at its C-terminus by a glycosylphosphatidylinositol membrane anchor. On average, each polypeptide carries carbohydrate chains composed of about 200 monosaccharide

units (galactose, *N*-acetylglucosamine, xylose, sialic acid, fucose, mannose and arabinose), which are most probably O-linked to hydroxy amino acids. The mucin-like molecules are the target of the fish's humoral immune response, but do not undergo antigenic variation akin to that observed for the variant surface glycoprotein in salivarian trypanosomes. The results are discussed with reference to the differences between natural and experimental infections, and in relation to the recently delineated molecular phylogeny of trypanosomes.

Key words: antigenic variation, evolution, GPI anchors, immune response.

INTRODUCTION

In comparison with the large amount of information that is available on the biochemistry, molecular biology and immunology of trypanosomes infecting mammals [1–6], very little is known about parasites of the genus *Trypanosoma* in other vertebrate classes. In analogy with the salivarian trypanosomes of mammals, the flagellates that infect birds [7], reptiles [8], amphibians [9] and fishes [10–12] are believed to live exclusively extracellularly, in the blood and tissue fluids of their hosts. The high abundance and apparent chronicity of trypanosome infections in non-mammalian vertebrates poses the question of how these parasites deal with the vertebrate's immune response and whether they have developed evasion mechanisms related to the antigenic variation observed in the Salivaria. We have approached this problem along two lines. In one project, we attempted to deduce the phylogeny of trypanosomes by analysing both rRNA genes and protein-coding genes of representative members of all vertebrate classes (see [13] and related work cited therein; J. Haag, P. Overath and C. O'hUigin, unpublished work). We concluded that trypanosomes are monophyletic and that the salivarian trypanosomes diverged early from all other trypanosomes. This suggested that antigenic variation of a proteinacious surface coat may be a unique invention of the salivarian lineage.

In a second project, we decided to study infections of the freshwater-fish trypanosome, *T. carassii*, in the carp as a model for a non-mammalian parasite/host pair. The principal replicating stages of this trypanosome are trypomastigotes in the vascular system of fishes and epimastigotes in the digestive tract

of leeches [10–12]. There is no evidence for an intracellular stage in the vertebrate host [14]. *T. carassii* infects a variety of cyprinid fishes, such as carp, goldfish, crucian carp and tench, as well as some members of non-cyprinid families, and is commonly found in the blood of fish populations in Nature. Prevalence in farmed fish may approach 100% [10,11,14]. In contrast, laboratory experiments in specified pathogen-free carp showed that *T. carassii* infections are effectively controlled by antibodies, and cross-protection between a cultured clone and derived lines from chronically infected carp excluded antigenic variation akin to that observed in salivarian trypanosomes as an evasion mechanism [2,14]. Therefore the high prevalence and intensity of trypanosome infections in fish reared under (semi)natural conditions can be attributed to their poor immune status.

This study presents the identification, purification and partial characterization of abundant, highly glycosylated mucin-like surface proteins of *T. carassii*, which are anchored in the plasma membrane by glycosylphosphatidylinositol (GPI) residues. The surface properties of this fish parasite thus appear to be very similar to those of *T. cruzi*, suggesting that a carbohydrate-dominated surface coat may be a general property of non-salivarian trypanosomes.

EXPERIMENTAL

Parasites and carp

Bloodstream forms of clone K1 of the carp-derived *T. carassii* isolate TsCc-NEM were grown as described previously [15].

Abbreviations used: CRD, cross-reacting determinant; GPI, glycosylphosphatidylinositol; GPI-PLC, GPI-specific phospholipase C; mAb, monoclonal antibody; NHS-LC-biotin, sulphosuccinimidyl 6-(biotinamido)hexanoate; VSG, variant surface glycoprotein.

¹ To whom correspondence should be addressed (e-mail peter.overath@tuebingen.mpg.de).

Procyclic cells of *T. brucei* Antat 1.1 were grown in semi-defined medium 79 [16]. Outbred carp (mean body weight 35.8 ± 4.1 g; 5 months old) were purchased from Bio International B.V., NL 6040 AP Roermond, The Netherlands.

Surface biotinylation

T. carassii bloodstream forms or *T. brucei* procyclic forms (pellets after centrifugation of 10^8 washed cells; 8000 g for 4 min) were resuspended in 200 μ l of cold PBS (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , pH 7.2). After addition of 2 μ l of NHS-LC-biotin [sulphosuccinimidyl 6-(biotinamido)-hexanoate (Pierce Chemical Co.); 100 mM in DMSO], the samples were incubated for 30 min on ice. Then, 2 μ l of 1 M Tris/HCl, pH 7.4, was added and the cells were collected by centrifugation (8000 g for 4 min). The pellets were taken up in 200 μ l of lysis buffer [50 mM Hepes, 2.5 mM EDTA, 2 mM EGTA, 10 μ M tosyl-lysylchloromethane ('TLCK'), 50 μ M leupeptin, pH 7.4]. After sonication (1–2 min), a membrane fraction was collected by centrifugation (110 000 g, 1 h, 4 °C). The membranes were washed once in 200 μ l of lysis buffer and then taken up in 167 μ l of buffer/33.3 μ l of 12% Triton X-114. After another high-speed centrifugation (110 000 g, 1 h), the pellet was suspended in 200 μ l of lysis buffer and the supernatant was subjected to phase separation [17]. The aqueous and detergent phases were adjusted to 200 μ l, and the detergent phases were extracted four times with toluene. After removal of residual toluene in a Speedvac, the volume was again adjusted to 200 μ l.

Cell fractionation and cleavage with GPI-specific phospholipase C (GPI-PLC)

A sonicated lysate (L) of *T. carassii* (13.8 mg of protein in 2 ml of lysis buffer) was centrifuged (110 000 g, 1 h), giving a membrane-containing insoluble fraction (M) and a soluble fraction (S). The membranes were washed once as described above and taken up in 200 μ l of water. Then 30 μ l of the membrane preparation was diluted with 30 μ l of 10 $\times$ GPI-PLC buffer (0.5 M Hepes, 25 mM EDTA, 5 mM dithiothreitol, pH 7.5), 240 μ l of water and 28 μ l of 12% Triton X-114, and incubated with or without 10 μ l of recombinant GPI-PLC from *T. brucei* (a gift from Dr. M. Carrington, Department of Biochemistry, University of Cambridge, U.K.; cf. [18]) for 2 h at 37 °C. After addition of a further 31 μ l of 12% Triton X-114, the samples were centrifuged (110 000 g, 1 h, 4 °C) and supernatants were subjected to phase separation as described above. This resulted in detergent-insoluble pellets (P), as well as aqueous (H_2O) and detergent (TX) phases, which were adjusted to the volume corresponding to the lysate (200 μ l). The gels shown in Figures 2–5 were loaded with fractions corresponding to 55.4 μ g of total cell protein.

Purification of mucin-like glycoproteins

A cell lysate (52 mg of protein in 10 ml of lysis buffer; 1.07 mg of protein corresponds to 10^8 cells) was processed to a washed membrane fraction as described above. The membranes were suspended in a solution containing 892 μ l of GPI-PLC buffer, 84 μ l of 12% Triton X-114 and 25 μ l of GPI-PLC. The sample was incubated overnight at 37 °C and then centrifuged at 110 000 g for 1 h at 4 °C. After addition of 108 μ l of 12% Triton X-114 and 0.2 μ l of Bromophenol Blue to the supernatant, the sample was subjected to phase separation. The aqueous phase was extracted again with 0.2 vol. of 12% Triton X-114, and the combined Triton phases were back-extracted once with 300 μ l of

PBS. The combined aqueous phases were dialysed overnight in the cold against 20 mM Tris/HCl, pH 8, concentrated in a Centriprep 10 Concentrator (Amicon, Beverley, MA, U.S.A.) to 500 μ l and then loaded on a 1 ml Mono-Q® HR5/5 column in an FPLC System (Amersham-Pharmacia, Freiburg, Germany). Bound proteins were eluted with a linear gradient from 0 to 1 M NaCl in 20 mM Tris/HCl, pH 8.0. Aliquots (2 μ l) of the fractions (1 ml) were tested by two dot-blots using monoclonal antibody (mAb) D5D5 and anti-(VSG 221) serum (where VSG is variant surface glycoprotein). Fractions 12–15, which were positive in both tests, were combined and transferred to 250 mM ammonium acetate using a Centriprep 10 Concentrator (final volume 0.5 ml). The sample was passed over a Superose® 12 HR10/30 gel filtration column taking 0.5 ml fractions, which were likewise probed on dot-blots with the two antibodies. Fractions 24–27, which were positive in both tests, were combined and the ammonium acetate was removed by several lyophilizations after the addition of water, yielding 449 μ g of product. A total of eight inositol determinations on two independently purified preparations yielded an average inositol content of 3.7 ± 0.9 pmol $\cdot$ μ g⁻¹.

Quantification of mucin-like glycoproteins by immunoblotting

Because the anti-(VSG 221) serum showed a negligible reaction before GPI-PLC treatment (see Figure 5), this antibody could be used for estimating the amount of mucin-like glycoproteins in a crude membrane preparation by immunoblotting. A washed membrane preparation from a lysate containing 26 mg of protein was resuspended in 446 μ l of GPI-PLC buffer and homogenized by sonication. After addition of 42 μ l of 12% Triton X-114, 25 μ l of 10 mM DTT and 12.5 μ l of GPI-PLC, the sample was incubated for 3 h at 37 °C, and then with a further 10 μ l of GPI-PLC for 9 h. Aliquots of 0.125, 0.25 and 0.5 μ l of undigested and digested samples, as well as standards of purified mucin-like proteins corresponding to 3.7, 7.4, 18.5 and 37 pmol of inositol, were subjected to immunoblotting [anti-(VSG 221) antibodies, 1:200, 3 h at room temperature and alkaline phosphatase-conjugated goat anti-(rabbit IgG) antibodies (1:2500, 2 h)]. The blot was developed using Nitroblue Tetrazolium chloride and disodium 5-bromo-4-chloro-3-indolyl phosphate. The alkaline phosphatase bound to the complex of mucin-like glycoproteins and anti-CRD antibodies (where CRD is cross-reactive determinant) was quantified by excising the corresponding areas from the blot and incubation of the slips in 1 ml of 10 mM *p*-nitrophenyl phosphate, 100 mM NaCl, 5 mM MgCl_2 and 100 mM Tris/HCl, pH 9.5. Plots of the absorbance at 405 nm for the PLC-treated samples (corrected for the untreated samples) and the standard samples (corrected for the value obtained for an irrelevant slip from the blot) against the amounts of extract or standard were linear, and allowed the estimation of the mucin-like glycoproteins in the extract by comparison.

Miscellaneous techniques

Electrophoresis in SDS/12%-polyacrylamide gels and immunoblotting to Immobilon-P® membranes (Millipore, Bedford, MA, U.S.A.) were performed as described previously [19]. Antibodies dissolved in 10 mM Tris/HCl, pH 7.9, 150 mM NaCl, 0.5% Tween 20 and 5% (w/v) milk powder were used at the following concentrations: rabbit anti-VSG [variant MITat 1.2 (221)], 1:200; rat mAb D5D5 hybridoma culture supernatant, 1:2; goat anti-(rabbit IgG) (code 111-055-003) or goat anti-(rat IgG) coupled to alkaline phosphatase (code 112-055-102; Jackson ImmunoResearch Laboratories), 1:2500. Silver staining of gels was performed by the method of Mayer [20].

Amino acid analysis was performed after hydrolysis of purified mucin-like glycoproteins (70 µg) in 100 µl of 6 M HCl containing 0.1% phenol *in vacuo* at 110 °C for 20 h. After evaporation of the acid, the dried amino acids were converted into the phenylthiocarbamyl derivatives and analysed by reverse-phase HPLC on a Pico-Tag column (Waters, Eschborn, Germany) using the conditions described previously [21]. Amino acid standard solutions (Sigma) were treated in the same way to correct for losses during hydrolysis. *myo*-Inositol analyses were performed by selected ion-monitoring GC-MS as described in [22], except that *myo*-[1,2,3,4,5,6-²H₆]inositol was used as an internal standard. Monosaccharides were analysed by GC-MS as methylglycoside trimethylsilyl derivatives, as described in [23].

RESULTS

Detection of GPI-anchored surface glycoproteins and GPI-anchored glycolipids in *T. carassii*

Bloodstream forms of *T. carassii* [15] and, as a control, procyclic cells of *T. brucei* were subjected to surface biotinylation using NHS-LC-biotin. Membrane fractions derived from these cells were extracted with Triton X-114. The detergent-insoluble proteins (P) and the aqueous (H₂O) and detergent (TX) phases obtained after phase separation of the detergent extract were analysed for biotinylated proteins on a blot (Figure 1). Whereas the aqueous phases gave rise to very little labelling, the detergent phase of *T. carassii* showed a broad biotinylated region corresponding to a molecular mass of 36–57 kDa and a sharp band at molecular mass of ~175 kDa. The 36–57 kDa proteins could not be detected by staining a polyacrylamide gel with Coomassie Brilliant Blue. As expected, the detergent phase of the procyclic cells contained the heterogeneously glycosylated procyclic acidic repetitive protein/procyclin containing Gly-Pro-Glu-Glu-Pro repeats [24,25] as the predominant biotinylated molecule.

A cell lysate (L) of *T. carassii* was fractionated into a soluble fraction (S) and an insoluble, membrane-containing fraction (M). Aliquots of the insoluble fraction were treated or not with *T. brucei* GPI-PLC in the presence of Triton X-114 (see the Experimental section). Centrifugation yielded a detergent-insoluble pellet (P) and a detergent extract. Proteins in the extracts were in turn partitioned by phase separation into aqueous

Figure 1 Labelling of surface proteins by biotinylation

Bloodstream forms of *T. carassii* or procyclic forms of *T. brucei* were labelled as described in the Experimental section. The figure shows a blot of membrane fractions probed with ExtrAvidin alkaline phosphatase and developed with Nitroblue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. P, proteins insoluble in Triton-114; H₂O and TX, aqueous and detergent phases respectively obtained after phase separation of the detergent extract. Aliquots equivalent to 8×10^5 cells were loaded in each lane.

Figure 2 Proteins detected by silver staining of cell fractions of *T. carassii*

Fractions were separated by SDS/PAGE and stained with silver. L, cell lysate (lane 1); S, soluble proteins (lane 2); M, insoluble, membrane-containing fraction (lane 3); P, Triton X-114-insoluble proteins (lane 4); H₂O and TX, aqueous and detergent phases respectively without (lanes 5 and 6) or with (lanes 7 and 8) GPI-PLC treatment. For further details, see the Experimental and Results sections.

Figure 3 Detection of carbohydrates in cell fractions of *T. carassii*

A blot of an SDS/polyacrylamide gel with the same loading as in Figure 2 was labelled with digoxigenin-3-*O*-succinyl- ϵ -aminohexanoic acid hydrazide using the DIG Glycan Detection Kit (Boehringer Mannheim).

(H₂O) and detergent (TX) phases. The fractions were analysed by PAGE and silver staining (Figure 2). As expected, the cell lysate and the soluble and insoluble fractions (lanes 1–3), as well as the detergent-insoluble pellet (lane 4), showed numerous components throughout the entire gel. Comparison of lanes 5–8 (aqueous and detergent phases) revealed a broad band in the 48–62 kDa region that partitioned into the detergent phase before GPI-PLC treatment and into the aqueous phase thereafter.

Figure 3 shows a blot of the same samples probed for the presence of carbohydrate by treatment with NaIO₄, digoxigenin-3-*O*-succinyl- ϵ -aminohexanoic acid hydrazide and anti-digoxigenin antibodies. Lane 7, corresponding to the aqueous phase after GPI-PLC treatment, revealed a strongly labelled region centred around a molecular mass of 50 kDa, while the detergent phase (lane 8) contained very little of these molecules. These components partitioned into the detergent phase before GPI-PLC treatment (compare lanes 5 and 6). A glycoconjugate with a molecular mass above 175 kDa was associated with the aqueous phase independent of GPI-PLC treatment (lanes 5 and 7). The molecules centred around a molecular mass of 50 kDa in lane 7 appeared to distribute over the entire upper half of the gel

Figure 4 Immunoblot of cell fractions from *T. carassii* probed with mAb D5D5

The loading of the gel was the same as in Figure 2.

Figure 5 Detection of GPI anchors by use of antibodies against the CRD (a) and sensitivity to digestion with proteinase K (b)

(a) A blot of the aqueous (H_2O) and detergent (TX) phases without (—PLC) and with (+PLC) prior treatment with GPI-PLC was probed with an anti-VSG antiserum. (b) Samples of $5 \mu l$ of detergent phase (not treated with GPI-PLC) in the presence or absence of $1.5 \mu l$ of proteinase K ($1 \text{ mg} \cdot \text{ml}^{-1}$) and $3.2 \mu l$ of $3 \times \text{SDS}$ -containing sample buffer were incubated for 5 h at 55°C and then subjected to immunoblotting using mAb D5D5. Lane 1, without proteinase K, lane 2, with proteinase K.

in the lysate (lane 1), the membrane fraction (lane 3) and the detergent extract (lane 6). Finally, the lysate, the membranes and the detergent extract contained weakly stained carbohydrate-containing components in the 10–25 kDa region of the gel, which could not be detected by immunoblotting after GPI-PLC treatment (lanes 7 and 8).

A similar picture (Figure 4) emerged when a blot of the same fractions was probed with mAb D5D5, which was shown previously by immunofluorescence experiments to react with the surface of *T. carassii* [15]. The lysate (lane 1), the membranes (lane 3) and the detergent extract (lane 6) showed intense staining in both the upper and the lower parts of the gel. After GPI-PLC treatment, the molecules centred in the 50 kDa region were found in the aqueous phase (lane 7), while the components in the low-molecular-mass region (10–25 kDa) were no longer detectable (compare lanes 7 and 6).

A blot of the fractions obtained by detergent-phase separation was probed with an anti-VSG antiserum as a source of antibodies reactive with the CRD exposed on GPI anchors after cleavage with GPI-PLC (Figure 5a; cf. [26]). Before enzyme treatment, no

Figure 6 Purification of mucin-like glycoproteins

Aliquots of the aqueous phase after Triton X-114 phase separation (H_2O) and the combined fractions after the Mono Q (anion exchange; AE) and the Superose 12 (gel filtration; GF) columns were subjected to immunoblotting using either mAb D5D5 (1×10^7 cell equivalents $\cdot \text{lane}^{-1}$) or anti-(VSG 221) antibodies (2×10^7 cell equivalents $\cdot \text{lane}^{-1}$), or stained in the gel by silver (2×10^7 cell equivalents $\cdot \text{lane}^{-1}$). Lanes 10 and 11 show silver staining of the glycoproteins obtained in two independent purifications ($12 \mu \text{g} \cdot \text{lane}^{-1}$).

Table 1 Amino acid and monosaccharide compositions of mucin-like glycoproteins from *T. carassii*

The values for amino acid analysis are the average of two determinations, and values for monosaccharide analysis are means $\pm$ S.D. of three determinations, except for arabinose and sialic acid (means of two determinations).

Amino acid	Composition (%)
Asx	2.3
Glx	4
Ser	11.2
Gly	14.5
His	0.7
Arg	0.8
Thr	22.3
Ala	10.6
Pro	7.2
Tyr	1.2
Val	8.7
Ile	2.9
Phe	3.3
Lys	3.5

Monosaccharide	Amount (residues per inositol residue)
Ara	12.2 ± 8.2
Fuc	16.3 ± 3.0
Xyl	20.6 ± 3.5
Man	13.2 ± 3.6
Gal	100 ± 17
Glc	Trace
GlcNAc	30.2 ± 9.6
Sialic acid	16.8 ± 7.7

positive protein was detectable (lanes 1 and 2), whereas after treatment the aqueous phase showed a broad band centred around 40 kDa (lanes 3 and 4). The detergent phase was treated with proteinase K and subjected to immunoblotting using mAb D5D5 (Figure 5b). Comparison of the two lanes demonstrated that components in the high-molecular-mass region were com-

Figure 7 Localization of antigens reactive with mAb D5D5 in trypanosomes embedded in Lowycryl HM20

Ultra-thin sections of *T. carassii* were treated with mAb D5D5 and 12 nm anti-rat IgG gold [27]. fp, flagellar pocket; fl, flagellum; n, nucleus; c, cytostome; g, Golgi stacks. Bar = 0.5 μ m.

pletely digested, whereas those in the low-molecular-mass region were resistant to proteinase K.

The results obtained so far can be summarized as follows. *T. carassii* contains a heterogeneous population of GPI-anchored glycoproteins with a molecular mass of 40–50 kDa; these are exposed on the cell surface, since they can be biotinylated with an amino-group-specific, impermeant reagent. The heterogeneity is likely to be caused by the number/size of the carbohydrate chains, because the bulk of the polypeptide(s) [as detected by biotinylation (Figure 1) or indirectly via their GPI anchor (Figure 5)] is centred around 40 kDa, whereas the bulk of the carbohydrate [as detected by a digoxigenin-tagged hydrazide (Figure 3) or by a carbohydrate-specific mAb (see Figure 4 and below)] is centred around 50 kDa. We will refer to these proteins as GPI-anchored mucin-like glycoproteins. In addition, the parasites contain heterogeneous GPI-anchored components of lower molecular mass which cannot be biotinylated (Figure 1), are resistant to the action of proteinase K and react strongly with the mAb. After cleavage with PLC, the released hydrophilic carbohydrates can no longer bind to the poly(vinylidene difluoride) membrane (Figure 4). We suggest that these components are GPI-anchored glycolipids.

Purification of the mucin-like glycoproteins

The mucin-like glycoproteins lacking the lipid anchor were purified from the aqueous phase on successive anion-exchange and gel-filtration columns and then analysed by immunoblotting using mAb D5D5 (Figure 6, lanes 1–3), anti-VSG antibodies (lanes 4–5) and silver staining (lanes 7–9). Lanes 11 and 12 of

Figure 6 show silver staining of samples obtained in two independent purifications. As judged by silver staining, the procedure yielded a product that was not significantly contaminated by other proteins and lacked the heterogeneous glycolipids. A minor co-purifying contaminant with a molecular mass of approx. 80 kDa could be detected in blots (cf. Figure 6, lane 3) and faintly in silver-stained gels (lane 10).

The number of molecules per cell was determined by an immunoblotting technique using a purified sample with a known inositol content as a standard. This yielded an estimate of $(6.0 \pm 1.7) \times 10^6$ molecules $\cdot$ cell $^{-1}$ ($n = 4$). Taking an average molecular mass of 55 000 Da and $2 \text{ mg} \cdot (10^8 \text{ cells})^{-1}$ for the cellular dry weight [i.e. about twice the amount of cell protein ($1.07 \text{ mg of protein} \cdot 10^8 \text{ cells}^{-1}$)], this copy number implies a cellular content of $54.8 \mu\text{g} \cdot 2 \text{ mg}^{-1}$ (2.74%). A typical purification yielded 439 μg from 104 mg dry weight, a yield of 15% in terms of the initial mucin-like glycoprotein content.

Compositional analysis

Table 1 shows the amino acid and monosaccharide compositions of the purified mucin-like glycoproteins. The proteins have a high content of threonine and serine residues, as well as glycine, alanine, valine and proline. The presence of one inositol residue is characteristic of GPI-anchored proteins. Based on the inositol content, one polypeptide chain carries carbohydrates containing, on average, 209.3 monosaccharide residues, including (in decreasing abundance) galactose, *N*-acetylglucosamine, xylose, sialic acid, fucose, mannose and arabinose. The N-terminus of the glycoproteins is blocked, and incubation with N-glycosidase

Figure 8 Reaction of carp IgM with mucin-like glycoproteins

The blot shows the reaction of 3 μ g of purified glycoproteins with naive carp IgM (lane 2; 2.7 μ g $\cdot$ ml⁻¹) or IgM from carp that had controlled an infection with *T. carassii* (lane 1; 3.6 μ g $\cdot$ ml⁻¹) probed with mAb WCI 12 (10% culture supernatant; cf. [28]) and alkaline-phosphatase-conjugated goat anti-(mouse IgG/IgM) (1:2500).

F did not change the apparent molecular mass in SDS/polyacrylamide gels (results not shown).

Immunoelectron microscopy using mAb D5D5

mAb D5D5 has been shown previously to react with the surface of *T. carassii* bloodstream forms in immunofluorescence experiments. The studies were refined here by immunogold labelling using this antibody. Strong labelling was observed at the plasma membrane, the flagellum and the flagellar pocket membrane (Figure 7a), as well as the cytostome (Figure 7b). Furthermore, the antibody prominently detected antigen associated with the Golgi stacks (Figure 7c).

Antigenicity of the mucin-like glycoproteins in carp

The mucin-like glycoproteins were immunogenic in carp, as shown by blotting experiments using IgM isolated from carp that had controlled an experimental infection (Figure 8, lane 1). In contrast, IgM from naive carp did not react at all (lane 2). As noted previously [14], immune IgM causes agglutination of trypanosomes. We also showed previously that immune IgM injected into carp protects against a challenge infection [14]. Our results here indicate that this protective effect can be mediated by antibodies binding to the parasite surface. Trypanosomes (10^8 in 4 ml of serum-free medium) were treated with 5 mg of immune or non-immune carp IgM (1 h at 0 °C) and then washed twice. In the former case, most cells were in aggregates, while in the latter case they were free. Each parasite preparation was then distributed into 10 carp (weight 35.8 ± 4.1 g) by intraperitoneal injection. After 1 week, the parasitaemia in carp infected with immune-IgM-treated parasites was $(3.4 \pm 4.6) \times 10^5$ trypanosomes $\cdot$ ml of blood⁻¹, while the control carp contained $(4.7 \pm 6.6) \times 10^6$ trypanosomes $\cdot$ ml of blood⁻¹, i.e. a 14-fold difference. The protection by surface-bound antibodies is probably not complete, because antibodies may dissociate from some cells before they are lysed by complement. Preliminary attempts to immunize carp with the purified mucin-like molecules (20 μ g in complete Freund's adjuvant per 50 g fish; booster immunization after 60 days with 10 μ g in incomplete Freund's adjuvant; bleeding after 20 days) showed that only one in ten fish had a detectable titre of antibody against a total trypanosome lysate. Therefore in this formulation these molecules are poorly immunogenic, while they elicit antibody formation during an active infection.

Figure 9 Comparison of clone K1, line 2 and line 3 by immunoblotting

Washed membranes were resuspended in GPI-PLC buffer containing 1% Triton X-114 and treated with GPI-PLC. Equal amounts of the digested samples (corresponding to 203 μ g of total cell protein) were probed on immunoblots with mAb D5D5 or anti-VSG antibodies as a source of anti-CRD antibodies. Lanes 1, 4 and 7, clone K1; lanes 2, 5 and 8, line 2; lanes 3, 6 and 9, line 3. Molecular mass markers on the left refer to lanes 1–6; markers on right refer to lanes 7–9.

Lack of change of mucin-like molecules during chronic infections

We have shown previously [14] that the extended persistence of trypanosomes in some laboratory-kept carp that have controlled the acute phase of an infection is not caused by antigenic variation, because clone K1 propagated in culture and two lines (lines 2 and 3, established after 107 days and 1 year respectively from carp that had controlled an infection with clone K1) were cross-protective. In the present study, the mucin-like proteins in clone K1 (Figure 9, lanes 1, 4 and 7), line 2 (lanes 2, 3 and 8) and line 3 (lanes 3, 6 and 9) were compared in their reactivity against mAb D5D5 and anti-CRD antibodies. No differences regarding staining intensity or mobility of the mucin-like proteins could be detected, indicating that no major changes had occurred.

DISCUSSION

The fish stage of *T. carassii* contains a family of mucin-like glycoproteins at a high copy number of approx. 6×10^6 molecules per cell. Their location at the cell surface is suggested (1) by their modification by a GPI anchor, (2) by their accessibility to an impermeant biotinylation reagent (Figure 1), (3) by their recognition by the monoclonal antibody D5D5, which labels the plasma membrane both in immunofluorescence [15] and in immunoelectron-microscopic experiments (Figure 7), and (4) by their reaction in cells and on blots with the lectin wheatgerm agglutinin (A. Lischke and P. Overath, unpublished work). The copy number is of the same order of magnitude as that of procyclic acidic repetitive protein/procyclin in the insect stage of *T. brucei* $\{(2.6 \pm 0.4) \times 10^6$ or 6×10^6 molecules per cell; cf. [25] and [29] respectively} and of the GPI-anchored mucins of metacyclic forms of *T. cruzi* ($> 1.5 \times 10^6$ copies per cell; cf. [30]).

The amino acid composition of the purified glycoproteins demonstrates a high content of threonine, glycine, serine, alanine, valine and proline residues. This is analogous to GPI-anchored mucins from the metacyclic [31], epimastigote [30,32] and trypomastigote [33] stages of the mammalian parasite *T. cruzi*, as well as secreted and membrane-bound mammalian mucins [34,35], which contain these same amino acids in abundance. The amino acid composition, as well as the lack of cleavage by N-glycosidase F, suggest that the fish trypanosome mucins are modified by

carbohydrate chains that are O-linked to hydroxy amino acids, but linkage by phosphodiester bonds is also a possibility. In *T. cruzi*, the mucin family is transcribed from hundreds of genes which predict a relatively conserved signal peptide, a hyper-variable region, Thr/Pro-rich repeated elements containing galactose-rich oligosaccharides linked via GlcNAc- α 1-O-Thr linkages and a relatively conserved C-terminus with a GPI anchor addition signal [36–39]. A closer comparison between the mucins of the two trypanosomes would require cloning of the genes from *T. carassii*, preferentially by immuno-screening of an expression library.

Based on the inositol content, the carbohydrate chains of the purified mucin-like molecules contain on average about 200 monosaccharide residues, composed of galactose, *N*-acetylglucosamine, xylose, sialic acid, fucose, mannose and arabinose. The structures of O-linked oligosaccharides from epimastigotes [31,32,40], metacyclics [31] and trypomastigotes [33] of *T. cruzi* have been determined. They are attached to the protein core via GlcNAc residues, which either remain unmodified or are substituted with up to five galactose and terminal sialic acid residues. The compositional analysis suggests that, in the *T. carassii* mucins, oligosaccharides could likewise be attached via GlcNAc, but are probably larger and more diverse in composition. By analogy with *T. cruzi*, we may assume that the fish parasites carry trans-sialidase at their surface, which transfers neuraminic acids from host serum components to the mucin-like glycoproteins. The negative charges are considered to make the parasites more resistant against lysis by the alternative complement pathway [41,42]. Arabinose has been found previously in trypanosomatids (uniquely as D-arabinopyranose) in the lipophosphoglycan of *Leishmania major* [43], the lipoarabinogalactan of *Crithidia fasciculata* [44] and the glycoinositol phospholipids of *Endotrypanum* spp. [45]. Xylose has been found in the glycoinositol phospholipids of *Herpetomonas samuelpessoai* [46] and, together with fucose, in the Gp72 glycoprotein of *T. cruzi* epimastigotes [47,48]. Thus there is precedent in other trypanosomatid glycoconjugates for all of the monosaccharides found in the *T. carassii* mucins.

mAb D5D5 is most probably directed against a carbohydrate epitope of the O-linked glycans, because the epitope it recognizes survives denaturation in SDS and is present on proteinase K-resistant glycolipids. The epitope is highly abundant in the Golgi stacks (Figure 7c), i.e. the cellular site for the synthesis of O-linked carbohydrates. From the Golgi, the glycoconjugates can be transported by vesicular flow to the flagellar pocket membrane, as well as possibly to the membrane lining the cytostome.

As a further analogy with *T. cruzi*, we detected a GPI-anchored glycolipid in membranes of *T. carassii*, which also carries the epitope recognized by mAb D5D5. In *T. cruzi*, the corresponding glycolipid is an inositol phosphoceramide substituted by a glycan structure, which is similar to that found in GPI anchors of proteins [49–52]. A more detailed comparison requires a structural analysis of the *T. carassii* glycolipid.

In summary, we suggest that *T. carassii* bloodstream forms are covered by extended, highly glycosylated and negatively charged proteins and similar glycosylated lipids which provide a protective layer against attack by the alternative complement pathway; therefore the parasite will survive in a non-immune host. This may be a general feature of trypanosomes parasitizing different vertebrate classes, with the exception of the salivarian trypanosomes that infect mammals, which are covered by a proteinaceous coat. It may be proposed that the ancestor of all extant trypanosomes carried a carbohydrate-rich surface similar to that found in *T. carassii* or *T. cruzi*. This carbohydrate-dominated coat may have been instrumental for parasite establishment in

aquatic or terrestrial invertebrates and initial invasion of the vascular system of vertebrates. The example of the, as far as we know, exclusively extracellular fish trypanosome [14] tells us that the prevalence and chronicity of infections in natural populations is not due to antigenic variation, but rather is a consequence of the limited effectiveness of the immune system of the host under the given environmental conditions. In fact, the prevalence and intensity of trypanosome infections may be a sensitive indicator of the immune status of natural vertebrate populations. In the case of *T. cruzi*, which likewise shows no antigenic variation of the kind observed in the *Salivaria* [53–55], the invasion of and replication in host cells provides an additional and probably secondarily acquired evasion mechanism that ensures the chronic course of the infection.

On this background, the emergence of salivarian antigenic variation poses a major problem. First, the *Salivaria* stand alone and diverged early in trypanosome phylogeny [13]. Thus no close relatives have been discovered so far which could possibly show a related phenomenon. Secondly, the complex mechanism of antigenic variation involving many hundreds of genes [2] can be imagined to have arisen by (1) selection through the vertebrate immune system, and (2) parasite cycling between vertebrate and the vector. How, in such a scenario, a highly immunogenic proteinaceous coat 'wins' against a poorly immunogenic carbohydrate-dominated surface remains a mystery.

This work was supported by the Deutsche Forschungsgemeinschaft and the Fond der Chemischen Industrie. M. A. J. F. is supported by a programme grant (054491) from The Wellcome Trust, and I. C. A. is supported by a grant (98/10495-5) from FAPESP-Brazil. We thank Dr. M. Carrington for providing GPI-PLC, Dr. Thomas Ilg for advice and critical reading of the manuscript, M. Bayer for help with some of the experiments, and Jens Ruoff and Kerstin Bär for expert technical assistance.

REFERENCES

- Vickerman, K., Myler, P. J. and Stuart, K. D. (1993) in *Immunology and Molecular Biology of Parasitic Infections* (Warren, K. S., ed.), pp. 170–212, Blackwell Scientific Publications, Boston.
- Cross, G. A. M. (1996) *BioEssays* **18**, 283–291.
- Molyneux, D. H. and Ashford, R. W. (1983) *The Biology of Trypanosoma and Leishmania: Parasites of Man and Domestic Animals*, Taylor and Francis, London.
- Viens, P. (1985) in *Immunology and Pathogenesis of Trypanosomiasis* (Tizard, I., ed.), pp. 201–223, CRC Press, Boca Raton.
- Albright, J. W. and Albright, J. F. (1991) *Parasitol. Today* **7**, 137–140.
- Takle, G. B. and Snary, D. (1993) in *Immunology and Molecular Biology of Parasitic Infections* (Warren, K. S., ed.), pp. 213–236, Blackwell Scientific Publications, Boston.
- Apanius, V. (1991) *Parasitol. Today* **7**, 87–90.
- Telford, Jr., S. R. (1995) in *Parasitic Protozoa*, vol. 10 (Kreier, J. P., ed.), pp. 161–223, Academic Press, New York.
- Bardsley, J. E. and Harmsen, R. (1973) *Adv. Parasitol.* **11**, 1–73.
- Lom, J. and Dyková, I. (1992) *Protozoan Parasites of Fishes*, Elsevier, Amsterdam.
- Lom, J. (1979) in *Biology of the Kinetoplastida*, vol. II (Lumsden, W. H. R. and Evans, D. A., eds.), pp. 269–337, Academic Press, New York.
- Woo, P. T. K. (1987) *Parasitol. Today* **3**, 186–188.
- Haag, J., O'hUigin, C. and Overath, P. (1998) *Mol. Biochem. Parasitol.* **91**, 37–49.
- Overath, P., Haag, J., Mameza, M. G. and Lischke, A. (1999) *Parasitology* **119**, 591–601.
- Overath, P., Ruoff, J., Stierhof, Y.-D., Haag, J., Tichy, H., Dykova, I. and Lom, J. (1998) *Parasitol. Res.* **84**, 343–347.
- Brun, R. and Schönenberger, M. (1979) *Acta Trop.* **36**, 289–292.
- Bordier, C. (1981) *J. Biol. Chem.* **256**, 1604–1607.
- Carnall, N., Webb, H. and Carrington, M. (1997) *Mol. Biochem. Parasitol.* **90**, 423–432.
- Wiese, M., Berger, O., Stierhof, Y.-D., Wolfram, M., Fuchs, M. and Overath, P. (1996) *Mol. Biochem. Parasitol.* **82**, 153–165.
- Mayer, A. (1995) *Proteintransport über die mitochondriale Außenmembran*. Verlag Korneli, München.
- Oxley, D. and Bacic, A. (1995) *Glycobiology* **5**, 517–523.
- Smith, R., Braun, P. E., Ferguson, M. A. J., Low, M. A. and Sherman, W. R. (1987) *Biochem. J.* **248**, 285–288.

- 23 Ferguson, M. A. J. (1994) in *Glycobiology: A Practical Approach* (Fukuda, M. and Kobata, A., eds.), pp. 349–383, IRL/Oxford University Press, Oxford
- 24 Bütikofer, P., Ruepp, S., Bschung, M. and Roditi, I. (1996) *Biochem. J.* **326**, 415–423
- 25 Treumann, A., Zitzmann, N., Hülsmeier, A., Prescott, A. R., Almond, A., Sheehan, J. and Ferguson, M. A. J. (1997) *J. Mol. Biol.* **269**, 529–547
- 26 Zamze, S. E., Ferguson, M. A. J., Collins, R., Dwek, R. A. and Rademacher, T. W. (1988) *Eur. J. Biochem.* **176**, 527–534
- 27 Stierhof, Y.-D., Schwarz, H., Menz, B., Russell, D. G., Quinten, M. and Overath, P. (1991) *J. Cell Sci.* **99**, 181–186
- 28 Secombes, C. J., van Groningen, J. J. M. and Egberts, E. (1983) *Immunology* **17**, 309–317
- 29 Clayton, C. E. and Mowatt, M. R. (1989) *J. Biol. Chem.* **264**, 15088–15093
- 30 Serrano, A. A., Schenkman, S., Yoshida, N., Mehlert, A., Richardson, J. M. and Ferguson, M. A. J. (1995) *J. Biol. Chem.* **270**, 27244–27253
- 31 Schenkman, S., Ferguson, M. A. J., Heise, N., Cardoso de Almeida, M. L., Mortara, R. A. and Yoshida, N. (1993) *Mol. Biochem. Parasitol.* **59**, 293–304
- 32 Previato, J. O., Jones, C., Xavier, M. T., Wait, R., Travassos, L. R., Parodi, A. J. and Mendonça-Previato, L. (1995) *J. Biol. Chem.* **270**, 7241–7250
- 33 Almeida, I. C., Ferguson, M. A. J., Schenkman, S. and Travassos, L. R. (1994) *Biochem. J.* **304**, 793–802
- 34 Devine, P. L. and McKenzie, I. F. C. (1992) *BioEssays* **14**, 619–625
- 35 Strous, G. J. and Dekker, J. (1992) *Crit. Rev. Biochem. Mol. Biol.* **27**, 57–92
- 36 Di Noia, J. M., Sánchez, D. O. and Frasch, A. C. C. (1995) *J. Biol. Chem.* **270**, 24146–24149
- 37 Di Noia, J. M., Pollevick, G. D., Xavier, M. T., Previato, J. O., Mendonça-Previato, L., Sánchez, D. O. and Frasch, A. C. C. (1996) *J. Biol. Chem.* **271**, 32078–32083
- 38 Di Noia, J. M., D'Orso, I., Aslund, L., Sánchez, D. O. and Frasch, A. C. C. (1998) *J. Biol. Chem.* **273**, 10843–10850
- 39 Previato, J. O., Sola-Penna, M., Agrellos, O. A., Jones, C., Oeltman, T., Travassos, L. R. and Mendoca-Previato, L. (1998) *J. Biol. Chem.* **273**, 14982–14988
- 40 Previato, J. O., Jones, C., Goncalves, L. P., Wait, R., Travassos, L. R. and Mendoca-Previato, L. (1994) *Biochem. J.* **301**, 151–159
- 41 Cross, G. A. M. and Takle, G. B. (1993) *Annu. Rev. Microbiol.* **47**, 385–411
- 42 Hall, B. F. and Joiner, K. A. (1993) *J. Eukaryotic Microbiol.* **40**, 207–213
- 43 McConville, M. J., Thomas-Oates, J. E., Ferguson, M. A. J. and Homans, S. W. (1990) *J. Biol. Chem.* **265**, 19611–19623
- 44 Schneider, P., Treumann, A., Milne, K. G., McConville, M. J., Zitzmann, N. and Ferguson, M. A. J. (1996) *Biochem. J.* **313**, 963–971
- 45 Xavier da Siveira, E., Jones, C., Wait, R., Previato, J. O. and Mendoca-Previato, L. (1998) *Biochem. J.* **329**, 665–673
- 46 Routier, F. H., da Siveira, E. X., Wait, R., Jones, C., Previato, J. O. and Mendoca-Previato, L. (1995) *Mol. Biochem. Parasitol.* **69**, 81–92
- 47 Ferguson, M. A. J., Allen, A. K. and Snary, D. (1983) *Biochem. J.* **213**, 313–319
- 48 Haynes, P. A., Ferguson, M. A. J. and Cross, G. A. M. (1996) *Glycobiology* **6**, 869–878
- 49 Previato, J. O., Gorin, P. A. J., Mazurek, M., Xavier, M. T., Fournet, B., Wieruszkes, J. M. and Mendonça-Previato, L. (1990) *J. Biol. Chem.* **265**, 2518–2526
- 50 de Lederkremer, R. M., Lima, C., Ramirez, M. I. and Casal, O. L. (1990) *Eur. J. Biochem.* **192**, 337–345
- 51 de Lederkremer, R. M., Lima, C., Ramirez, M. I., Ferguson, M. A. J., Homans, S. W. and Thomas-Oates, J. E. (1991) *J. Biol. Chem.* **266**, 23670–23675
- 52 Carreira, J. C., Jones, C., Wait, R., Previato, J. O. and Mendoca-Previato, L. (1996) *Glycoconjugate J.* **13**, 955–966
- 53 Snary, D. (1980) *Exp. Parasitol.* **49**, 68–77
- 54 Plata, F., Pons, F. G. and Eisen, H. (1984) *Eur. J. Immunol.* **14**, 392–399
- 55 Eisen, H. and Kahn, S. (1991) *Curr. Opin. Immunol.* **3**, 507–510

Received 6 September 1999/5 November 1999; accepted 16 November 1999