Structural Analysis and Antibody Response to the Extracellular Glutathione S-Transferases from *Onchocerca volvulus*

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Received 2 March 2001/Returned for modification 11 June 2001/Accepted 23 August 2001

Onchocerca volvulus is a human pathogenic filarial parasite which, like other parasitic nematodes, is capable of surviving in an immunologically competent host by employing a variety of immune evasion strategies and defense mechanisms including the detoxification and repair mechanisms of the glutathione S-transferases (GSTs). In this study we analyzed the glycosylation pattern and the immunological properties of extracellular *O. volvulus* GST1a and -1b (OvGST1a and -1b). The enzymes differ in only 10 amino acids, and both are glycoproteins that have cleavable signal peptides and unusual N-terminal extensions. These characteristics have not been described for other GSTs so far. Mass spectrometry analyses indicate that both enzymes carry high-mannose type oligosaccharides on at least four glycosylation sites. Glycosylation sites 1 to 3 of OvGST1a (OvGST1b sites 2 to 4) are occupied by truncated N-glycans (Man₂GlcNAc₂ to Man₅GlcNAc₂), and N glycosylation site 4 of OvGST1a (OvGST1b site 5) carries Man₅GlcNAc₂ to Man₆GlcNAc₂. To analyze the capacity of these secretory GSTs to stimulate host immune responses, we studied the antibody responses of onchocerciasis patients against the native affinity-purified OvGST1a and -1b. By enzyme-linked immunosorbent assay we showed that OvGST1a and -1b are immunodominant antigens, with less than 7% nonresponder patients. A direct comparison of the antibody responses to the glycosylated and deglycosylated forms demonstrates the high immunogenicity of the N-glycans. Analyses of the antibody responses to the unusual N-terminal extension show an enhanced recognition of this portion by patients as opposed to recognition of the recombinant protein without extension.

Onchocerca volvulus is the causative agent of onchocerciasis, a disease that affects about 20 million people in Africa, the Arabian Peninsula, and Central and South America. The control of this disease is largely dependent on administration of annual doses of ivermectin, a drug that is able to kill the microfilariae but not the adult worms (22).

The spectrum of *O. volvulus* infection varies from asymptomatic microfilaridemia often associated with immunological hyporesponsiveness to severe skin and eye diseases including onchodermatitis and blindness. Establishment of infection and disease development are dependent on the specific antigen recognition and immune response of the host. The complex immune response is triggered by numerous different filarial antigens at the same time. To elucidate the mechanism of the immune response, it is essential to explore the specific reactions to individual, native parasite antigens.

The glutathione S-transferases (GSTs) are a family of detoxification enzymes that catalyze the conjugation of reduced glutathione (GSH) to xenobiotic and endogenous electrophilic compounds (25, 49, 55, 56). Besides having a role in detoxification, they are involved in the protection of tissues against oxidative damage and in the intracellular transport of hydrophobic substrates as noncatalytic carrier proteins (ligandins) (for recent reviews see references 14, 26, and 52). In a parasitic

context it is especially important to consider their function in the regulation of the stress response, in the detoxification of lipid peroxidation products, in drug resistance, and possibly in the modulation of the host immune defense mechanisms (8, 16, 42).

Several studies demonstrate the participation of filarial GSTs in the defense against oxidative stress (34, 35). The potential to protect the parasite against host immunity makes them attractive candidate vaccine antigens (11). The significant differences between the tertiary structure of the helminth GSTs and that of the host enzymes make the GSTs promising chemotherapeutic targets (9).

From the perspective of antibody reactivity, the most immunogenic filarial antigens that have been identified include paramyosin, tropomyosin, OvALT-1, SXP1, and chitinase (1, 18, 21, 30). The ease of recombinant production of these and other potential vaccine molecules has made vaccination trials using various animal models possible. However, as a result of this, the role that carbohydrates and other nonprotein determinants play in the immune responses to parasites has been undervalued. However, it is well recognized that most of the immunodominant antigens either are excreted or secreted proteins or are abundant constituents of the nematode surface that are glycosylated (36, 38).

The *O. volvulus* GST1a and -1b (OvGST1a and -1b) are unique GSTs in that they are glycoproteins possessing signal peptides that are cleaved off in the process of producing the mature protein. The mature protein starts with a 25-amino-acid extension not present in other GSTs. The ultrastructural

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localization of the secretory OvGST1a and -1b in parts of the cuticle and in the outer lamellae of the hypodermis is consistent with the fact that they are secretory proteins (31). The structures of the N-glycans have been determined, and a three-dimensional model has been created to demonstrate their localization profile. By virtue of the potential significance of OvGST1a and 1b as immunoprophylactic targets, we have concentrated our efforts on the immunological relevance of the N-glycans and of the uncommon N-terminal extension.

(This work was conducted by A. Sommer in partial fulfillment of the requirements for a PhD from the University of Hamburg, Hamburg, Germany.)

MATERIALS AND METHODS

Parasites, preparation, and purification of native OvGST1a and -1b. Nodules containing *O. volvulus* female adults were removed from untreated patients with generalized onchocerciasis in Benin, as described previously (2). Nodulectomies for research purposes were approved by the Ethics Commission of the Medical Board Hamburg. Adult worms were homogenized on ice with a glass and glass potter in phosphate-buffered saline (PBS), pH 7.4, containing 0.1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged for 1 h at 100,000 × g, and the native OvGSTs were purified from the supernatant as previously described (32).

Production of recombinant OvGST1a and -1b. The different OvGST constructs were cloned into expression vector pJC20 and transformed in *Escherichia coli* strain BL21. Expression of recombinant OvGST1a (rOvGST1a) and -1b, followed by purification using GSH-Sepharose, was done as previously described (33).

Site-directed mutagenesis. To obtain the mutation of M25 to A25 in OvGST1a, PCR was performed with the specific sense oligonucleotide 5'-GCTACAACCTCA AGCGGAAAAGTACACATTAAC-3' and antisense oligonucleotide 5'-GTATA TGTGTACTTTTCCGCTTGAGGTTGTAGC-3' using 1:100 (vol/vol) OvGST1a/pJC40 as the template. Following PCR using *Pfu* polymerase, the products were incubated with *DpnI* for 1 h at 37°C and 1/10 (vol/vol) was transformed in *E. coli* strain DH5α.

Synthesis of two 17-mer peptides of the N-terminal extension. The following overlapping peptides of the N-terminal extension, coupled to poly-L-lysine, were synthesized at IPF PharmaCeuticals GmbH: (ASSNANQAITSINKP)₈K₇A and (AITSINKPKGKLOPQ)₈K₇A.

Modeling, model refinement, and structure validation. Three-dimensional models were generated based on the crystal structure of the squid sigma class GST (PDB code: 1GSQ) (19). The primary amino acid sequences of OvGST1a and the squid GST have a similarity of 45%, with 25% identical amino acids. The modeling of the three-dimensional structure of OvGST1 followed a standard stepwise procedure, starting with an alignment of the target sequence onto the template structure using the Malign program. Due to the missing N-terminal 25 amino acids in the template, this stretch of amino acids was omitted in the modeling steps. The initial model was generated by the MODELLER4 package (48), and a first energy minimization step was performed using the CHARMM force field (7). Following the superpositioning of the protein structures, refinements were carried out with the AMBER force field (45). Subsequently, the energy minimization was performed with 100 steps of steepest descent followed by 100 steps of a quasi-Newton minimization (46) to alleviate steric clashes between atoms and obtain a rational peptide geometry. The quality of the model was assessed using different validation tools. The OvGST1a structure was validated with the program PROCHECK (29) at each step of the model building. No significant geometric violations were detected in the final model. Molecular visualization was carried out with the programs MOLSCRIPT and Raster3D (41).

Deglycosylation of the native OvGST1a and -1b. The native OvGSTs were dialyzed against 10 mM EDTA–50 mM sodium phosphate, pH 7.5, heated at 80°C for 2 min, and then incubated overnight with the appropriate amount of N-glycosidase F at 37°C.

The endoglycosidase H digestion was performed in 20 mM sodium acetate, pH 5.8, as described above. The deglycosylation of OvGST1a and -1b was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were revealed by silver staining or by the use of the Glyco-Track kit (Oxford Glycosystems) in accordance with the manufacturer's instructions.

ELISA. The reactivity of the N-glycans to patient sera was assessed by an enzyme-linked immunosorbent assay (ELISA). The optimal coating concentra-

tion of the recombinant and native antigens and serum dilutions were determined by a checkerboard titration ELISA using a pool of sera from *O. volvulus*-infected individuals. Following dialysis against 0.1 M sodium carbonate buffer, pH 9.6, the glycosylated and deglycosylated native OvGSTs were covalently coupled to activated CovaLink NH plates (Nunc) at a concentration of 1 µg/ml. The rOvGSTs were used at a final concentration of 2 µg/ml. The antigens were incubated overnight at room temperature, washed three times with distilled water, and then blocked with 5% (wt/vol) dry milk–PBS, pH 7.5. All sera were used at a dilution of 1/400 (vol/vol). Bound antibodies were detected with peroxidase-conjugated antibodies against human immunoglobulin G (IgG) using tetramethylbenzidine as the substrate. Between each incubation step, five washing cycles with PBS–16.9 g of NaCl/liter–10 g of MgCl₂/liter–0.05% Tween 20 were performed. After the substrate reaction was stopped by 0.5 M H₂SO₄, the optical density at 450 nm (OD₄₅₀) was measured. Sera from confirmed microfilaria-positive patients with generalized onchocerciasis (38 samples) were obtained from Benin and Guinea (5, 6). Negative controls included experiments using irrelevant proteins for coating and experiments performed without coating or serum. To exclude the influence that preheating of OvGST1a and -1b before deglycosylation has on antibody recognition, comparative ELISA of the native proteins was performed with and without preheating and overnight incubation at 37°C. African control sera (four samples) and sera from Europeans not exposed to filariae (five samples) were used to determine the cutoff level. As a positive control, one test serum of a patient with generalized onchocerciasis with known high antibody concentration was included on each test plate, and only intra- and interassay variations less than 10% were accepted.

For the analysis of the cross-reactivity of the N-terminal extension, the following sera of confirmed *O. volvulus*-negative persons infected with different helminths were used: *Schistosoma mansoni*, three individual sera; *Loa loa*, three sera; *Trichinella spiralis*, four pools with three sera per pool; *Ascaris lumbricoides*, five pools with three sera per pool; *Brugia malayi* and *Wuchereria bancrofti*, each five pools with two sera per pool.

Statistical analysis. Differences between the data in two groups were analyzed by the Mann-Whitney U-test and the paired and unpaired Wilcoxon test. A *P* value of <0.05 was regarded as significant.

Preparation of native OvGST1a and -1b for mass spectrometry (MS) analysis. Purified native OvGST1a and -1b were dialyzed against 10 mM Tris-HCl, pH 8.1, followed by heating at 80°C for 2 min. The proteins were incubated overnight at 37°C with trypsin at a 30:1 ratio in 100 mM Tris-HCl, pH 8.1–0.1 mM dithiothreitol–0.02% (wt/vol) Na₂S₂O₃. Following a second dialysis against 10 mM ammonium hydrogencarbonate, pH 7.4, the peptide mixture was lyophilized and stored at –20°C until use.

HPLC and ESI-MS/MS. The tryptic peptide mixtures (approximately 100 pmol) were analyzed on an Applied Biosystems 172Å microbore high-pressure liquid chromatography (HPLC) system using an Aquapore OD-300 C₁₈ column (1.0 by 100 mm) at a flow rate of 40 µl/min and a linear gradient from 4 to 56% acetonitrile in 0.1% formic acid and 4 mM ammonium acetate. Elution of peptides was monitored by UV absorption at 214 nm and by MS on a TSQ 700 triple-quadrupole instrument equipped with a Finnigan ES ion source connected on line to the HPLC system. In a preparative run, (glyco)peptide fractions were collected manually and subjected to Edman sequencing (Applied Biosystems; model 475A). Matrix-assisted laser desorption/ionization–time of flight (MALDI-TOF) MS and electrospray ionization tandem mass spectrometry (ESI-MS/MS) analysis are described below.

MALDI-TOF MS. For analysis by MALDI-TOF MS the (glyco)peptide fractions were measured using a matrix of 19 mg of α-cyano-4-hydroxycinnamic acid in 400 µl of acetonitrile and 600 µl of 0.1% (vol/vol) trifluoroacetic acid in water. The sample solution was mixed with the same volume of the respective matrix. The concentration of the analyte mixture was 1 to 10 pmol/µl. Determination of the molecular masses was carried out by positive-ion MALDI-TOF MS using a Bruker REFLEX time-of-flight instrument as described by Grabenhorst et al. (15).

ESI-MS/MS. For analysis by ESI-MS/MS the (glyco)peptide samples (approximately 3 µl) were used to fill gold-coated nanospray glass capillaries (Protana, Odense, Denmark). The tip of the capillary was placed orthogonally in front of the entrance hole of a quadrupole time-of-flight mass spectrometer (Micromass, Manchester, United Kingdom) equipped with a nanospray ion source, and a voltage of approximately –1,000 V was applied. For collision-induced dissociation experiments, parent ions were selectively transmitted from the quadrupole mass analyzer into the collision cell. Argon was used as the collision gas, and the kinetic energy was set at around –35 eV. The resulting daughter ions were then separated by an orthogonal time-of-flight mass analyzer (50).

FIG. 1. Purification and deglycosylation of native *OvGST1a* and *-1b*. The proteins were separated on an SDS-12.5% PAGE gel. Lane A, *OvGST1a* and *OvGST1b* at 31 to 33 and about 34 kDa, respectively. The relatively broad bands can be explained by N-glycan microheterogeneity. For comparison, r*OvGST1a* raised in *E. coli* was also applied (lane B). Lane C, completely deglycosylated proteins after N-glycosidase F treatment; lane D, digestion with endoglycosidase H, leaving a single GlcNAc residue at each glycosylation site in place. Left, migration positions of molecular mass standard proteins.

RESULTS AND DISCUSSION

Comparison of the amino acid sequences of *OvGST1a* and *OvGST1b*. The purification of glutathione-binding proteins from *O. volvulus* extract, produced by centrifugation at $100,000 \times g$, by GSH affinity chromatography yielded three proteins of roughly 34, 31, and 26 kDa, representing *OvGST1b*, *OvGST1a*, and *OvGST2*, respectively (31, 32). The average content of purified *OvGST1a* and *-1b* was 0.5 μg /adult female worm. Only the fractions containing native *OvGST1a* and *-1b* were used for further experiments. Figure 1, lane A, demonstrates the purification of

native *OvGST1a* and *-1b*. Due to the microheterogeneity of the N-glycans, a broader band between 31 and 33 kDa, representing *OvGST1a*, is seen whereas *OvGST1b* is seen as the upper, very faint band. Immunoblot analysis using *O. volvulus* extract and anti-r*OvGST1* demonstrates the same staining pattern (31). This strongly indicates that the observed difference in the quantities of *OvGST1a* and *-1b* is not due to the purification procedures, i.e., different affinities for binding to GSH-Sepharose, but rather reflects the situation in the worm.

To unequivocally demonstrate the presence of two different isoforms of *OvGST1*, the affinity-purified native *OvGSTs* were tryptically digested and analyzed by HPLC-MS for the abundances of two theoretical cleavage products (A and B) of *OvGST1a* and *OvGST1b*, differing only in one amino acid (A: $_{99}\text{FGLLG}\text{TND}[\text{A}]\text{WEEAK}_{111}$; B: $_{112}\text{IMAVVLNID}[\text{E}]\text{ELFQK}_{125}$; amino acid of *OvGST1b* is in brackets). The identity of the expected peptide fragments was demonstrated by ESI-MS/MS peptide sequencing of the respective fractions collected from the preparative HPLC run (data not shown). The ratio between *OvGST1a* and *-1b* found by this method was approximately 7:1.

Nucleotide differences found in the respective cDNAs for *OvGST1a* and *-1b* result in 10 structural changes (Fig. 2). Southern blot and sequence analyses demonstrate that *OvGST1a* and *-1b* are encoded by separate genes. The observed nucleotide differences are confirmed by sequence analysis of the respective genomic copy (E. Liebau et al., unpublished results). These amino acid differences are found mainly in the N-terminal region of the mature protein, with 4 out of 10 amino acid changes located in the N-terminal extension. The replacement of polar residues with more-hydrophilic, charged residues

FIG. 2. Alignment of the amino acid sequences of *OvGST1a* and *OvGST1b*. The signal peptide, comprising 25 amino acids, is indicated. Arrows, cleavage site, N-terminal extension, and tryptic cleavage sites. The numbering of the respective amino acids begins at position 1 (in boldface), which corresponds to the signal peptide cleavage site of the mature protein. The numbers above the sequences correspond to the tryptic fragments shown in Table 1. The differences between *OvGST1a* and *-1b* are in boldface.

TABLE 1. Molecular weights of the tryptic fragments of *Ov*GST1a and -1b analyzed by HPLC-MS^a

Tryptic peptide	HPLC peak no.	Amino acid position in the mature protein	<i>Ov</i> GST1a			<i>Ov</i> GST1b		
			Calculated peptide mass (Da)	Detected peptide signal ([M + nH] ⁿ⁺)	Deduced peptide mass (Da)	Calculated peptide mass (Da)	Detected peptide signal ([M + nH] ⁿ⁺)	Deduced peptide mass (Da)
1	5	1–18	1,858.9	930.5 ²⁺	1,859.0			
1a		1–3				304.18	n.d. ^e	
1b		4–18 ^c				2,903.84	n.d.	
2		19–20	203.1	n.d.		275.15	n.d.	
3	3	21–27	872.4	873.2 ¹⁺	872.2	900.45	n.d.	
4	11	28–36	1,133.6	567.9 ²⁺	1,133.8	Identical to 1a		
5		37–38	231.1	n.d.		Identical to 1a		
6	1	39–43	586.3	587.3 ¹⁺	586.3	Identical to 1a		
7	13	44–57 ^c	2,840.3	1,421.2 ²⁺	2,840.4	Identical to 1a		
8		58–60	388.24	n.d.		Identical to 1a		
9	13 ^f	61–64	576.3	577.4 ¹⁺	576.4	Identical to 1a		
10	17 (Mox. ^f)	65–98 ^c				4,844.4	1,615.7 ³⁺	4,844.1
10a	1	65–69	675.4	676.2 ¹⁺	675.2			
10b	15 (Mox.), 16	70–98 ^c	4,230.0	1,411.0 ³⁺	4,230.0			
11	13	99–111	1,478.7	740.3 ²⁺	1,478.6	1,434.7	718.3 ²⁺	1,424.6
12	14	112–125	1,631.9	817.0 ²⁺	1,632.0	1,645.9	823.9 ²⁺	1,645.8
13	8	126–133	1,022.56	1,023.6 ¹⁺	1,022.6	Identical to 1a		
13 + 14 ^d	6	126–137 ^c	2,683.2	1,342.7 ²⁺	2,683.4	Identical to 1a		
15		138	146.1	n.d.		Identical to 1a		
16	9	139–143	634.3	635.2 ¹⁺	634.2	Identical to 1a		
17	12	144–156 ^c	2,680.0	1,341.2 ²⁺	2,680.4	2,679.1	1,340.7 ²⁺	2,679.4
18		157–159	438.2	n.d.		Identical to 1a		
17 + 18 ^d	13	144–159 ^c	3,100.3	1,551.2 ²⁺	3,100.4	3,099.4	n.d.	
19	2	160–162	406.3	407.2 ¹⁺	406.2	Identical to 1a		
20	2	163–173	1,141.6	571.9 ²⁺	1,141.8	Identical to 1a		
21	18	174–193	2,239.1	1,120.6 ²⁺	2,139.2	Identical to 1a		
22	12	194–206	1,536.8	769.3 ²⁺	1,536.6	Identical to 1a		
23	7	207–214	842.5	843.3 ¹⁺	842.3	Identical to 1a		
24	4	215–218	574.3	575.3 ¹⁺	574.3	Identical to 1a		
25		219	146.11	n.d.		Identical to 1a		
26		220–222	399.26	n.d.		Identical to 1a		
27	10	223–225	429.2	430.1 ¹⁺	429.1	Identical to 1a		

^a Monoisotopic theoretical masses and masses deduced from multiply charged ions ([M + n H]ⁿ⁺) detected by ESI-MS are given.

^b The tryptic peptide numbering corresponds to that in Fig. 2. For better assignment, the amino acid positions of the tryptic fragments are also given. *Ov*GST1a- and -1b-specific fragment, resulting from *Ov*GST1a- and -1b-specific cleavage sites, are indicated by "a" and "b".

^c Glycopeptide. The associated masses are those of the peptide and its major glycoform, Man₅GlcNAc₂.

^d Incomplete tryptic digestion.

^e n.d., not detected; peptides with masses of <400 Da were not detected by the mass spectrometer.

^f Mox., oxidized methionine.

changes the character of the N-terminal extension of *Ov*GST1b from a neutral one to a more hydrophilic one.

Isolation and characterization of N-linked oligosaccharides.

As previously shown (31), the native enzymes are extensively glycosylated. The glycosylation accounts for approximately 13 and 20% (*Ov*GST1a and *Ov*GST1b, respectively) of their apparent masses (Fig. 1, lane A). For comparison the nonglycosylated protein expressed in *E. coli* (Fig. 1, lane B) as well as the fully deglycosylated peptide backbone after *N*-glycanase F treatment (Fig. 1, lane C) were run on the same SDS-PAGE gel. To characterize the glycan structure of the purified native proteins, *Ov*GST1a and -1b were subjected to tryptic digestion. There were 27 tryptic cleavage sites for both *Ov*GSTs, with one specific cleavage site for each protein. Cleavage between amino acids 3 and 4 in *Ov*GST1b resulted in specific fragments 1a and 1b, and cleavage between amino acids 69 and 70 of *Ov*GST1a generated specific fragments 10a and 10b (Fig. 2; Table 1). The resulting fragments were separated by microbore

HPLC, and the elution was monitored by UV absorption and MS. Figure 3 shows the corresponding UV trace at 214 nm. The peaks identified by HPLC-MS analysis are marked (Fig. 3; Table 1); further peaks correspond to nonpeptide contaminants as well as to trypsin fragments. Most of the nonglycosylated tryptic peptides were identified; peptides <400 Da, however, were not detected in the HPLC-MS analysis (Fig. 3 and Table 1). All *Ov*GST1a peptides bearing a glycosylation site were identified by the characteristic pattern of molecular ions due to the attachment of different glycans (as shown for glycopeptides 1 and 4 in Fig. 4). On account of the small quantity of *Ov*GST1b and the minute differences between both proteins observed, only two specific glycopeptides of *Ov*GST1b were identified. Glycopeptide 1, consisting of amino acids 4 to 18 (GP1_{4–18}) of *Ov*GST1b, could not be detected and analyzed (Table 1). On all four glycosylation sites of *Ov*GST1a, molecular ions with mass increments of 162 Da were detected, suggesting the presence of high-mannose type structures differing

peak no.	tryptic peptide (aa)	peak no.	tryptic peptide (aa)	peak no.	tryptic peptide (aa)	peak no.	tryptic peptide (aa)
1	39 - 43; 65 - 69	6	126 - 137*	11	28 - 36	16	70 - 98*
2	160 - 162; 163 - 173	7	207 - 214	12	144 - 156*; 194 - 206	17	65 - 98* (Mox.)
3	21 - 27	8	126 - 133	13	44 - 57*; 61 - 64; 99 - 111; 144 - 159*	18	174 - 193
4	215 - 218	9	139 - 143	14	112 - 125		
5	1 - 18	10	223 - 225	15	70 - 98* (Mox.)		

FIG. 3. Tryptic peptide mapping of the native *Ov*GST1a and *Ov*GST1b. The protein preparation was digested with trypsin as described in Materials and Methods. Tryptic fragments were separated by microbore HPLC, and each distinct peak was subjected to amino acid sequence analysis. For the identification of glycosylated peptides, aliquots from the fractions (asterisks) were separately analyzed for sugar constituents. Mox., oxidized methionine; RT, retention time.

in the number of mannose residues attached, as shown in Fig. 4 for GP1₄₄₋₅₇ and GP4₁₄₄₋₁₅₆. The assignment and the ratios of the glycan structures found at individual glycosylation sites of *Ov*GST1a are as follows: for GP1₄₄₋₅₇, Man₅/Man₄/Man₃ ratio = 1:0.32:0.1; for GP2₇₀₋₉₈, Man₅/Man₄/Man₃ ratio = 1:0.51:0.07; for GP3₁₃₄₋₁₃₇, Man₅/Man₄/Man₃ ratio = 1:0.13:0.07; for GP4₁₄₄₋₁₅₆, Man₅/Man₆/Man₇/Man₈/Man₄/Man₃/Man₉ ratio = 1:0.48:0.15:0.1:0.05:0.04:0.01. This demonstrates that Man₅GlcNAc₂ is the dominant glycoform present at all four glycosylation sites of *Ov*GST1a. Besides identifying this glycoform as dominant, the analysis revealed that about 30% of the N-glycans found are smaller (Man₃ and Man₄) than those described for mammals. Interestingly, whereas the structures of Man₂GlcNAc₂ to Man₅GlcNAc₂ and the relative ratios of oligosaccharides linked to glycosylation sites 1 (N50), 2 (N79), and 3 (N134) appear to be rather similar, at glycosylation site 4 (N144) clearly larger structures, bearing Man₅GlcNAc₂ to Man₉GlcNAc₂, were detected. For further characterization of the (glyco)peptides, individual fractions from another HPLC run were collected and the amino acid sequences of all glycopeptides were confirmed by Edman sequencing. Additionally, the MALDI-TOF MS spectrum shows the protonated molecular ions of tryptic GP1₄₄₋₅₇ bearing two to five mannose residues (Fig. 4). Small amounts of a tryptic

peptide with one missing cleavage site incorporating glycosylation site 4 (N144) were detected; this peptide bears high-mannose type glycans (Man₅ to Man₉) much larger than those found for the first glycosylation site (Fig. 4). This confirms the HPLC and ESI-MS/MS results for this site obtained for the completely digested GP4₁₄₄₋₁₅₆. The major components of the collected HPLC fractions were further characterized by ESI-MS/MS experiments. The daughter ion spectrum of doubly charged GP1₄₄₋₅₇ showed an abundance of fragment ions generated by the cleavage of monosaccharide bonds, as is explained in the fragmentation scheme of Fig. 5. In particular, the successive elimination of five hexose residues indicates the presence of a high-mannose type glycan. Peptide sequence-specific fragments from the amino and carboxy termini were much weaker but nevertheless allowed the confirmation of the peptide sequence. The other major glycopeptides were characterized in an analogous way.

The helminth's surface and excreted or secreted antigens represent the major challenge to the host's immune system and may be a key to the successful defense strategies of the parasite. Immunodominant epitopes are often accessible to periodate oxidation and/or are susceptible to peptide *N*-glycosidase F digestion. Numerous lectin binding studies confirm the prevalence of saccharide determinants on the parasite surface.

FIG. 4. Upper mass range of the MALDI-TOF spectrum of HPLC fraction 13 (compare Fig. 3) isolated by reverse-phase HPLC from *Ov*GST1a following tryptic digestion. The peptide containing glycosylation site 1 (N50) (left site) bears truncated mannose type glycans with two to five mannose residues (M_2 to M_5), whereas the peptide with one missing cleavage site and containing glycosylation site 4 (N144) (right site) bears high-mannose glycans with five to eight mannose residues. From the dominant component at m/z 2841.7, a daughter ion spectrum was obtained by ESI-MS/MS as depicted in Fig. 5.

However, only a few helminth carbohydrates have actually been structurally defined (13, 37).

The oligomannosyl structures that we found for *Ov*GST1a and -1b are common features in nematodes, as are truncated

glycans with one or two additional mannoses attached to the chitobiose core (13). Structural analysis of N-glycans of whole-worm extract from the filarial parasite *Acanthocheilonema viteae* displays high levels of N-glycans that contain phospho-

FIG. 5. ESI daughter ion spectrum of doubly charged GP1 with five mannose residues. The major doubly charged fragment ions are due to the elimination of one to five mannose residues. Complementary glycan fragments obtained by the cleavage of the chitobiose core and the subsequent loss of the mannose residues were obtained as indicated in the fragmentation scheme. Peptide-specific fragments incorporating the carboxy-terminal (y_n) or amino-terminal (b_n) amino acids are marked in the spectrum. GN, *N*-acetylglucosamine; M, mannose.

rylcholine (PC). These PC-glycans are also found in glycoproteins that are secreted by adult filarial parasites during parasitism in their final host. The PC component has been shown to interfere with key signal transducers implicated in cellular activation and proliferation and represents a novel target for chemotherapy (12). The N-glycans usually have trimannosyl cores that have one to four *N*-acetylglucosamine residues added and that either were or were not fucosylated. Besides the PC-glycans, a second family of N-glycans that are remarkably rich in GlcNAc have been found in filarial nematodes (17). Furthermore, complex and hybrid structures are also major constituents. These may have antennae that are truncated to a single GlcNAc, as was observed for *Dirofilaria immitis*, or nontruncated antennae that commonly have a backbone composed of laciNAc (GalNAc β 1-4GlcNAc) (24).

Although there still is no specific identification of a particular helminth glycoconjugate in mediating a specific host response, glycoconjugates are being increasingly implicated in the immune responses to parasites. Here they can be key modulators or targets of the host immune systems, and often the immunodominant epitopes are glycans of unique structures (13).

Homology modeling of OvGST1. Alignment of OvGST1a and -1b with GSTs from various classes demonstrates the relationship to the sigma class enzymes, particularly the hemopoietic prostaglandin D synthase from rats (23), one of the products of the GST gene families in the housefly *Musca domestica* that is involved in pesticide resistance (57), the S-crystallins constituting the major lens proteins in squids (54), and the GSTs isolated from squid digestive glands (19).

To analyze the localization of the N-glycans, a three-dimensional model of OvGST1a was prepared based on the X-ray crystallography data of the sigma class GST from squids (1GSQ) (Fig. 6). An alignment obtained with the program Blast2, version 2.1.1 (August 2000) indicates an overall similarity of 45%, a sequence identity of 25%, and only four gaps (2%). There are no large deviations from the empirical values; the OvGST1 model compares favorably with the structure of the squid GST. Due to the missing homology to the N-terminal extension, the model begins at position Q22. The overall topology of the OvGST1 monomer is similar to the typical GST structure and, as described for the squid sigma class GST, also is arranged in a smaller α/β domain (domain I) and a larger α domain (domain II). Domain I encompasses the first one-third of the enzyme and is built up in a $\beta\alpha\beta\alpha\beta\alpha$ structural motif that forms a mixed four-strand β sheet in the order of 4-3-1-2, with strand 3 antiparallel to the other three sheets. The overall fold of domain I is classified as being part of the thioredoxin superfamily fold (4, 39) and contains the principal determinants for GSH binding. Domain II of the protein is composed of five α -helices (α 4 to α 8) (Fig. 6). While domain I is mainly responsible for GSH binding, domain II provides the primary structural elements associated with the second substrate specificity (49).

The other known OvGST with glutathione-binding capacity (OvGST2) shows a strong topological relationship with the pi class GSTs (34). However, in OvGST2, the C-terminal coil lies to one side of the active site. This coil normally forms the back face of the hydrophobic substrate binding pocket, and its displacement results in a "tunnel-like" hole (34). Direct compar-

ison of the active sites of OvGST1 and OvGST2 shows that that of OvGST1 is more flattened, resulting in a wider and shallower cleft. This probably reflects the observed differences in the substrate specificities, with OvGST1 preferring bulky aromatic substrates.

The most striking difference between OvGST1 and other described GSTs is the N glycosylation. Four N-linked glycosylation sites were located at positions N50, N79, N134, and N144, corresponding to GP1₄₄₋₅₇, GP2₇₀₋₉₈, GP3₁₃₄₋₁₃₇, and GP4₁₄₄₋₁₅₆ of OvGST1a, respectively (Fig. 6). For OvGST1b an additional glycosylation site is located in the N-terminal extension (N6). Glycosylation site 1 (OvGST1b: glycosylation site 2) is found in a loop following α -helix 1, glycosylation site 2 (OvGST1b: glycosylation site 3) is located in the β -sheet 3, and glycosylation site 3 (OvGST-1b: glycosylation site 4) lies in a loop made out of five amino acids between α -helix 4 and α -helix 5. Finally, glycosylation site 4 (OvGST1b: glycosylation site 5) is located in α -helix 5 near a small loop region made out of two residues (E147 and S148) (Fig. 6). This demonstrates that the N-glycans cover, more or less, the entire enzyme. An additional binding site for nonsubstrate ligands and their glutathione conjugates was observed in the region of the crystallographic twofold axis, incorporating the α 4-turn- α 5 motifs of the two subunits in the sigma class GST from squid digestive glands (20). In the 26-kDa GST from *Schistosoma japonicum*, a third binding site for the antischistosomal drug praziquantel has been found near the dimer interface (40). The binding regions of the nonsubstrate binding sites of OvGST1a and -1b appear to be shielded by the N-glycans of glycosylation site 3 (N134), as well as by the large carbohydrate moieties of glycosylation site 4 (N144).

No difference in the binding of the glycosylated enzymes and that of the deglycosylated enzymes to the immobilized glutathione was observed by affinity chromatography, indicating that the N-glycans do not interfere with the GSH-binding site. Furthermore, no variance in substrate conjugation capacity between the glycosylated and deglycosylated forms was observed, demonstrating that the N-glycans do not interfere with the active site and have no measurable influence on the enzymatic activity. Comparison of the conjugation capacity of the recombinant OvGST1a plus N-terminal extension and that of the shortened form (without N-terminal extension) using universal substrate 1-chloro-2,4-dinitrobenzene (CDNB) shows no difference in the activities (data not shown). This, however, was expected, due to the topological distance between the active site and extension.

Influence of N glycosylation on the recognition of OvGST1a and -1b by patient sera. To analyze the influence of the partially truncated N-glycans on the recognition of OvGST1a and -1b by the immune system of the host, 29 sera of patients with generalized onchocerciasis were tested by ELISA (Fig. 7). For the deglycosylation, the native OvGST1a and -1b were treated with *N*-glycanase F (Fig. 1, lanes A and C). Deglycosylation was additionally performed using endoglycosidase H (Fig. 1, lane D). This experiment was necessary to analyze the influence of the altered charge due to the replacement of amino acid N with D, caused by treatment with *N*-glycosidase F. The IgG antibody response to endoglycosidase H-deglycosylated OvGST1a and -1b was similar to the antibody responses measured following *N*-glycosidase F treatment. Furthermore, com-

FIG. 6. N-glycosylation sites and the topology of the *Ov*GST1a monomer. Shown is a ribbon presentation of a three dimensional model of *Ov*GST1a based on the structure of the squid sigma class GST (PDB code: 1GSQ). α -Helices are red, and β strands are yellow. The N-to-C direction of the structural elements can be deduced by the labeling of the secondary structures. The locations of the N glycosylation sites are orange.

parative ELISA with native *Ov*GST1a and -1b with and without reheating and overnight incubation at 37°C was performed to exclude the influence of this pretreatment in the following ELISA measurements. Serological responses to native glycosylated and deglycosylated *Ov*GST1a and -1b are indicated in Fig. 7. Levels of *Ov*GST1a- and -1b-specific antibodies of non-infected individuals were analyzed using five European and four African control sera. In these sera, there was no recognition of the glycosylated and deglycosylated forms. The OD₄₅₀ cutoff of 0.18 can probably be attributed to conserved GST epitopes. The OD₄₅₀ values representing the IgG antibody responses to the *Ov*GSTs are significantly higher (paired test; $P < 0.05$) for the native glycosylated form, i.e., the native structure that is found in the living parasite (median OD₄₅₀ = 0.66 [10th and 90th percentiles, 0.32 and 0.79, respectively]), than for the deglycosylated form (median OD₄₅₀ = 0.36 [10th

and 90th percentiles, 0.19 and 0.58]) (Fig. 7). Using *E. coli*-expressed r*Ov*-GST1a instead of the deglycosylated protein also led to lower IgG responses than those for the native protein (data not shown). Out of the 29 onchocerciasis patient sera tested, 2 sera did not recognize the enzymes and 2 others recognized only the glycosylated form of the enzymes.

Epitope mapping of the *S. mansoni* 28-kDa GST identified three major antigenic sites (3). An alignment of *S. mansoni* 28-kDa GST with *Ov*GST1a reveals that three out of the four N-glycan sites are located directly in the antigenic regions. The high immunogenicity of the N-glycans of *Ov*GST1 and their location in homologous sites of confirmed antigenic epitopes indicate that these epitopes are of importance for the antigenicity of *Ov*GST1. These results indicate that posttranslational modifications of proteins clearly have an important influence with respect to their recognition by the host immune system.

FIG. 7. ELISA of the IgG response of human onchocerciasis infection sera to native glycosylated and deglycosylated *OvGST1*s. Twenty-nine sera of patients infected with *O. volvulus* were tested ($n = 3$). The responses were significantly higher ($P < 0.05$) for the glycosylated form (*OvGST1* plus CHO) than for the deglycosylated (*OvGST1* without carbohydrates) protein. The IgG responses of the patient sera (OD_{450} values) are given as individual points. Boxes, 50% area of total IgG antibody responses to *OvGST* with and without CHO; bars, 10th and 90th percentiles. The line marks the cutoff value.

Carbohydrates that are expressed by pathogens may incite several types of innate immune activation. When they are excretory-secretory antigens or are surface associated, they are recognized by antibodies or lymphocytes inducing various kinds of responses. They have been shown to bind to the serum mannose-binding lectin or to the mannose receptor on cells, facilitating complement binding and/or phagocytosis as part of the innate immune system (10, 51). Moreover it has been demonstrated that mannosylation of peptides enhances the potency to stimulate major histocompatibility complex class II-restricted T-cell clones, leading to selective targeting and superior presentation on dendritic cells (53). In addition, carbohydrates have the ability to activate the alternative pathway of complement. The ability of the carbohydrates on *OvGST1a* and -1b to induce or prime host T-cell responses is currently being investigated (A. Hoerauf et al., unpublished data).

Antibody recognition of the N-terminal extension of *OvGST1a*. When expressing the *OvGST1a* and -1b in *E. coli*, we get initiation of translation at two different methionines, resulting in the respective expression of proteins that differ in size. Whereas the 27-kDa protein has a 25-amino-acid N-terminal extension, the smaller protein does not (Fig. 2). This extension shows no homology to known GST structures. Searching the available databases did not result in any homologies to known peptide structures. By using the program ANTIGENIC (<http://www.uk.embnnet.org/Software/EMBOSS/>) based on the

method of Kolaskar and Tongaonkar (28, 44), we identified eight potential antigenic determinants in *OvGST1a* (A39-S52, I112-W129, G167-M184, A90-L102, L194-K206, F72-N79, S81-S88, and E147-F160) and one located in the N-terminal extension of the GST (K18-Q24). Based on this tentative information we analyzed the N-terminal extension of the GST in order to identify the antigenic potency of this *OvGST1*-specific region. To examine the antibody response to the N-terminal extension, 38 sera of patients with generalized onchocerciasis were tested by ELISA. For that purpose, the 27-kDa *OvGST1a* with N terminus (generated by mutating the second translation initiation start point from M25 to A25) was expressed and purified in order to preserve the conformational epitopes of the total protein. For comparison, we also expressed *OvGST1a* without the N terminus. Significantly higher antibody levels ($P = 0.046$) were found for *OvGST1a* plus the N terminus (median $OD_{450} = 0.28$ [10th and 90th percentiles, 0.16 and 0.58, respectively]) than for the N-terminally truncated protein (median $OD_{450} = 0.22$ [10th and 90th percentiles, 0.17 and 0.35, respectively]). Whereas four of the patient sera did not recognize the 27-kDa *OvGST1a* plus N-terminal extension, nine did not recognize the smaller 24.5-kDa protein. These results show that 34 out of 38 (89.5%) patient sera recognized the recombinant *OvGST1a* plus N-terminal extension as the antigen. From the patient sera that recognized both, i.e., the protein with and without the N-terminal extension, 22 out of 38 sera (58%) showed an enhanced reactivity to the protein with the N-terminal extension.

Reactivity of the N-terminal portion of *OvGST1a* with sera from patients with other helminth infections. All helminth GSTs have some distinct structural elements that lead to cross-reactivity with sera from patients infected with other helminths. For example, antibodies raised against affinity-purified *OvGSTs* react strongly with their *Brugia pahangi* and *Brugia malayi* counterparts (47). *OvGST1a* and -1b, however, each have a unique N-terminal extension not found in other GSTs so far. On account of the immunoreactivity observed, we synthesized two overlapping peptides comprising the N-terminal extension of *OvGST1a* and analyzed their cross-reactive properties with sera from patients infected with other helminths (*L. loa*, *B. malayi*, *W. bancrofti*, *A. lumbricoides*, *T. spiralis*, and *S. mansoni*). All of the tested sera showed low IgG responses, with OD_{450} values ≤ 0.15 . Twelve out of 20 sera from patients with onchocerciasis showed IgG antibody responses to the N-terminal extension of *OvGST1a*, with OD_{450} values > 0.1 , and 8 of these sera reacted weakly with the two peptides (median $OD_{450} = 0.2$ [10th and 90th percentiles, 0.052 and 0.86, respectively]; cutoff $OD_{450} = 0.04$). None of the sera (serum pools) from patients with filarial infections with *L. loa* or *B. malayi* exceeded OD_{450} values of > 0.1 . Two of five serum pools from patients infected with closely related filarial nematode *W. bancrofti* showed an IgG response to the peptides (OD_{450} values of > 0.1). This higher IgG reaction may be due to similar GST antigen epitopes, which might resemble the N-terminal portion of *OvGST1a*. As shown for the lymphatic filariasis-causing nematode *B. malayi*, three GSTs similar to *OvGST1* and *OvGST2* cross-react with anti-*OvGST* rabbit antisera, indicating that *B. malayi* possesses similar GSTs (47). The investigation of serum pools that were obtained from patients with non-filarial nematode infections demonstrates

that all four pools from *T. spiralis* infections showed IgG responses <0.1 while one of five pools from *A. lumbricoides*-infected patients showed a higher IgG reaction ($OD_{450} = 0.12$) with the N-terminal extension of OvGST1a. This may be due to a hidden or former infection with *O. volvulus*. The serum pools from patients infected with the trematode *S. mansoni* did not react with the N-terminal extension peptides (OD_{450} values of <0.1).

There is a need to expand our knowledge of the diversity and structure of N-glycans in filarial parasites. Carbohydrate antigens of the parasite are targets of humoral immunity and may play a role in modulating host immune responses. They may provide protective immunity against infection. Furthermore, carbohydrates might play an important role in mediating specific parasite defense or survival strategies by protecting extracellular proteins from proteolytic degradation or by suppressing certain immune responses, host lectin binding, and cell targeting. In the vertebrate host, glycoproteins are recognized by antibodies, mannose-binding proteins, and cellular mannose receptors. This recognition, in turn, represents an effective defense mechanism leading to complement fixation, opsonization, and activation of specific T- and B-cell responses against the parasite. Better understanding of these glycans and the immunity to them might have important implications for the design of immunization protocols in order to induce or enhance protective cell-mediated and humoral immunity in humans. A comparative study of glycosylated and nonglycosylated secretory 20-kDa retinol binding proteins from *O. volvulus* (Ov20), *B. malayi* (Bm20), and *A. viteae* (Av20) revealed three N-linked glycosylation sites for Ov20 and Av20 and one different site for Bm20, which may reflect functional differences (43). Ov20 and Av20, in contrast to Bm20, were strongly recognized by sera from patients with onchocerciasis but not from patients with lymphatic filariasis. The different glycosylation that was observed in the three different glycoproteins was discussed as being a contribution to the differential immunological reactivities found (43).

The analysis of proteins in their native state has become a prerequisite for a variety of functional and structural studies, including vaccine development. In the nematocidal vaccines tested so far, the *E. coli*-expressed candidate vaccine antigens confer substantially less protection than their native purified counterparts or no protection (27). The structure and immunological properties of the *E. coli*-expressed antigens differ from those of native worm antigens. The different structures, caused by the missing eucaryotic modifications, result in immune responses which are inefficient for killing the worm. This demonstrates the importance of analyzing posttranslational modifications, such as glycosylation of immunodominant antigens.

ACKNOWLEDGMENTS

This project is supported by the Deutsche Forschungsgemeinschaft (DFG projects Li793-1-4). P. Fischer was supported by the scholarship program "Infectiology" of the BMBF.

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Editor: S. H. E. Kaufmann