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A Novel Type of Glutathione *S*-Transferase in *Onchocerca volvulus*

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***Onchocerca volvulus* is a pathogenic human filarial parasite which, like other helminth parasites, is capable of evading the host's immune responses by a variety of defense mechanisms which are likely to include the detoxification and repair mechanisms of the enzyme glutathione *S*-transferase (GST). In this study, we show that one of the previously described GSTs from *O. volvulus* appears to possess the characteristics of a secreted enzyme. When the complete *O. volvulus* GST1 (OvGST1) sequence presented here is compared with those of other GSTs, 50 additional residues at the N terminus are observed, the first 25 showing characteristics of a signal peptide. This is consistent with the N-terminal sequence data on the native mature enzyme which begins at amino acid 26, based on the deduced protein sequence from the cDNA. The native protein, without the signal peptide sequence, possesses a 24-amino-acid extension not present in other GSTs. The deduced amino acid sequence of the OvGST1 cDNA clone was shown to possess four potential N-glycosylation sites. Digestion of *O. volvulus* homogenate with endoglycosidase, followed by detection of OvGST1 with specific antibody, indicated that the enzyme possesses at least two N-linked oligosaccharide chains. Gel filtration of the *Escherichia coli*-produced recombinant OvGST1 showed that it is enzymatically active as a nonglycosylated dimer. OvGST1 is found in the media surrounding adult worms maintained in culture, indicating that, in vitro, this enzyme is released from the worm. The strongest immunostaining for OvGST1 was observed in the outer cellular covering of the adult worm body, the syncytial hypodermis, especially in the interchordal hypodermis, where the peripheral membrane forms a series of lamellae which run into the outer zone of the hypodermal cytoplasm.**

Glutathione *S*-transferases (GSTs) are a group of multifunctional proteins encoded by a multigene family. They perform functions ranging from catalyzing the detoxification of electrophilic compounds to protecting against peroxidative damage (for recent reviews, see references 2, 10, and 30). The GSTs are ubiquitous among eukaryotes and have been found in a wide range of parasitic helminths (6), in which they have been shown not only to play a housekeeping role but also to protect against membrane damage induced by the cytotoxic products of lipid peroxidation, such as lipid hydroperoxides and reactive carbonyl species. These are secondary products of lipid peroxidation generated by host immune cell effector molecules (7, 23).

In mammals, four distinct cytosolic classes of GSTs (alpha, mu, pi, and theta) and a microsomal GST class (24) have been identified. The helminth GST cDNAs described so far encode proteins which show sequence similarity to different classes but cannot be clearly placed into any one class. In trematodes, the 28-kDa GSTs (4, 29) from *Schistosoma* spp. show sequence similarity to the alpha class, and the *Fasciola hepatica* (25) and the 26-kDa (15) GSTs from *Schistosoma* spp. show similarity to the mu class. All nematode GST cDNA sequences found up to this point show some degree of sequence identity to the pi class GSTs (*Caenorhabditis elegans* [34], *Ascaris suum* [19], and *Onchocerca volvulus* GST2 [21]), as is also the case with the *O. volvulus* GST1 (OvGST1) described here (20). However, OvGST1 also has novel features which have, to our knowledge, not yet been described for GSTs in general.

Immunization studies with the 28-kDa GST of *Schistosoma mansoni* (SM28 GST) and with *F. hepatica* GSTs (28) have demonstrated the immunoprotective properties of these enzymes and thus their potential as vaccine candidate antigens. Immunization with SM28 GST resulted in up to a 50% reduction of parasitic load in rats, mice, and hamsters (3, 4). Baboon immunization with SM28 GST markedly affected both worm viability and fecundity (5). Studies using GST to immunize sheep against *F. hepatica* showed a 57% reduction of worm burden (28). On the basis of the essential function that GSTs have in helminth parasites, as well as the success observed with GSTs as vaccine candidate molecules for schistosomiasis and fascioliasis, the *O. volvulus* GSTs will be tested as components of a vaccine against onchocerciasis.

This report presents data which show that OvGST1 is a glycoprotein which possesses a hydrophobic N-terminal signal peptide that is cleaved off in the process of producing the mature enzyme. This is followed by a stretch of residues which are not commonly found in other GSTs but are present in the native OvGST1 protein. The enzyme is active as a nonglycosylated dimer and is released by adult worms maintained in vitro. Immunolocalization by light microscopy indicates that OvGST1 plays a role near the host-parasite interface.

(This article is based on a doctoral study by Eva Liebau in the Faculty of Biology, University of Hamburg, Hamburg, Germany.)

MATERIALS AND METHODS

Reagents. The 5′ RACE (rapid amplification of cDNA ends) kit, restriction endonucleases, and RPMI 1640 were obtained from GIBCO BRL Life Technologies (Gaithersburg, Md.); the TA Cloning system was from Invitrogen (Heidelberg, Germany); *N*-glycosidase F was from Boehringer (Mannheim, Germany); and the Sequenase Version 2.0 sequencing kit was purchased from United States Biochemical Corp. (Cleveland, Ohio). Alkaline phosphatase–anti-alkaline phosphatase and the anti-mouse polyclonal antibody were from Dako Diagnos-

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tika GmbH (Hamburg, Germany). All other chemicals were reagent grade and were obtained from Sigma (St. Louis, Mo.) or Merck (Darmstadt, Germany).

Parasites. Subcutaneous nodules (onchocercomata), which contained adult *O. volvulus*, were removed by nodulectomy. Nodulectomies were performed in several African countries on untreated patients with generalized onchocerciasis, as described previously (1). Nodulectomies for research purposes were approved by the Ethics Commission of the Medical Board Hamburg.

Nodules containing the parasites were isolated and shipped to the laboratory on ice. By microdissection, the worms were isolated from the nodules and, to eliminate host tissue, incubated in RPMI 1640 containing 0.5% collagenase (*Clostridium histolyticum*). The collagenase digestion was carried out for 24 h in a 34°C shaking incubator; the end volume of RPMI 1640-collagenase per nodule was about 5 to 10 ml. Media were changed every 8 to 10 h, and finally, the worms were checked microscopically for purity from host material and viability.

Preparation of RNA and 5′ RACE. Adult *O. volvulus* worms were homogenized in guanidinium thiocyanate. The homogenate was layered on a CsCl step gradient, and the RNA was prepared as previously described (8).

To obtain the complete 5′ end of the mRNA and the sequence of the N-terminal portion of OvGST1, the RACE method was used. First-strand cDNA synthesis was primed with the gene-specific antisense oligonucleotide 549 (5′TACG GTTGTCTTCGTA3′). Following cDNA synthesis and subsequent purification, a homopolymeric dC tail was attached to the 3′ end of the cDNA. Tailed cDNA was amplified with the nested gene-specific primer 840 (5′GTTAATGTGTACTTT TCCAT3′) and a complementary homopolymer-containing anchor primer, permitting amplification from the homopolymeric tail. To increase the specificity, a second nested PCR was performed, using oligonucleotide 841 (5′ATTTGAGGTTGT AGCTTTCC 3′) and the anchor primer. The positions of the three primers are indicated in Fig. 1A. The PCR fragment obtained was ligated into pCR II, using the TA Cloning system, and the nucleotide sequence was determined.

In vitro maintenance of *O. volvulus*. The worms, incubated in RPMI 1640 under a gas phase of 5% O_2–5% CO_2–90% N_2, could survive for up to 4 weeks (16). The worms were maintained in 2 ml of medium. This medium was changed daily, and the spent medium with the secreted products was collected for 5 days. The production and the release of lactate were monitored daily for more than 2 weeks as a parameter of viability. The spent medium was concentrated by ultrafiltration (Centricon).

Preparation of parasite extract. Adult worms were homogenized with a glass/glass homogenizer in phosphate-buffered saline–1% Triton X-100 (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) containing 0.1 mM phenylmethylsulfonyl fluoride. The homogenates were centrifuged at 100,000 × *g* for 1 h.

Deglycosylation. The *O. volvulus* 100,000 × *g* supernatant was supplemented with sodium dodecyl sulfate (SDS) to make the final concentration 1% and was heated at 100°C for 10 min. The samples were mixed 1:4 with digestion buffer containing 200 mM sodium phosphate-1% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS)–10 mM EDTA, pH 8.6. The endoglycosidase peptide *N*-glycosidase F was then added from diluted stocks containing 2.5 mM EDTA, and the mixture was incubated at 37°C overnight.

Production of OvGST1 rabbit polyclonal antiserum. The recombinant antigen rOvGST1 was expressed in *Escherichia coli* and purified by affinity chromatography as previously described (21). Rabbit polyclonal antiserum was raised by subcutaneous immunization with 70 μg of rOvGST1 emulsified in Freund's complete adjuvant (day 0), followed by three immunizations (70 μg [each] in Freund's incomplete adjuvant: days 14, 28, and 56). Western blot (immunoblot) analysis with total *O. volvulus* protein extracts showed that the antiserum produced is specific for OvGST1 (and does not cross-react with OvGST2).

SDS-PAGE and Western blot. A 16- by 18-cm gel was used in order to enhance detection of protein size differences. After aliquots of *O. volvulus* 100,000 × *g* supernatant or 50 μl of 50×-concentrated medium was heated at 95°C for 10 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer, the proteins were separated by SDS–12.5% PAGE and electrophoretically transferred to a nitrocellulose membrane. OvGST1 was visualized by immunostaining, using a rabbit anti-rOvGST1 primary antiserum, anti(rabbit immunoglobulin G)-alkaline phosphatase-labelled antibody conjugate, and the chromogenic substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Immunolocalization of OvGST1. Twenty-five onchocercomata were fixed in 4% buffered formaldehyde or 80% ethanol and embedded in paraffin by standard methods. For immunohistochemistry, the alkaline phosphatase-anti-alkaline phosphatase method was applied according to the recommendations provided by the manufacturer and as described by Cordell et al. (9). As primary antibody, rabbit polyclonal antibody against rOvGST1 was used at a dilution of 1:1,000. Preimmune serum was used as the control at the same dilution. As secondary antibody, anti-rabbit monoclonal antibody and anti-mouse polyclonal antibody were applied. Fast Red TR salt was used as the chromogen, and hematoxylin functioned as the counterstain. The best labelling was achieved on ethanol-fixed sections.

Gel filtration chromatography and assay of the recombinant OvGST1 activity. Fast protein liquid chromatography was performed on a High Load Superdex 75 column (16 by 1.6 cm; Pharmacia). The column was equilibrated with 50 mM Tris-HCl (pH 8)–150 mM NaCl. rOvGST1 was loaded onto the column at a flow rate of 60 ml h^{-1}, 2-ml fractions were collected, and the metabolizing activity towards CDNB was determined (22).

Nucleotide sequence accession number. The completed OvGST1 mRNA has been assigned EMBL accession number X75029.

RESULTS

Analysis of the N terminus of OvGST1. The 5′ portion of OvGST1 mRNA was amplified by using the 5′ RACE procedure. The translation initiation site was assigned to the first ATG triplet. The nucleotide sequence surrounding this initiation codon agrees with the Kozak consensus sequence in that there is a purine in position −3 and a G in position +4 (17).

The start of the mature OvGST1 enzyme, as determined by N-terminal amino acid sequencing, lies at residue 26. The preceding 25 amino acids represent a signal peptide which is cleaved off by an endoproteinase (Fig. 1A). Figure 1B shows the hydrophobicity plot averaged over 10 residues. The hydrophobicity profiles of OvGST1 and OvGST2 have similarities beginning at residue 50 of OvGST1 and residue 1 of OvGST2.

OvGST1 has a typical signal sequence that can be divided into three subregions (32). It has a polar, positively charged amino-terminal part (n-region) (histidine at position 8), followed by an overall hydrophobic central stretch of amino acids (h-region) and, finally, a hydrophobic but more polar region

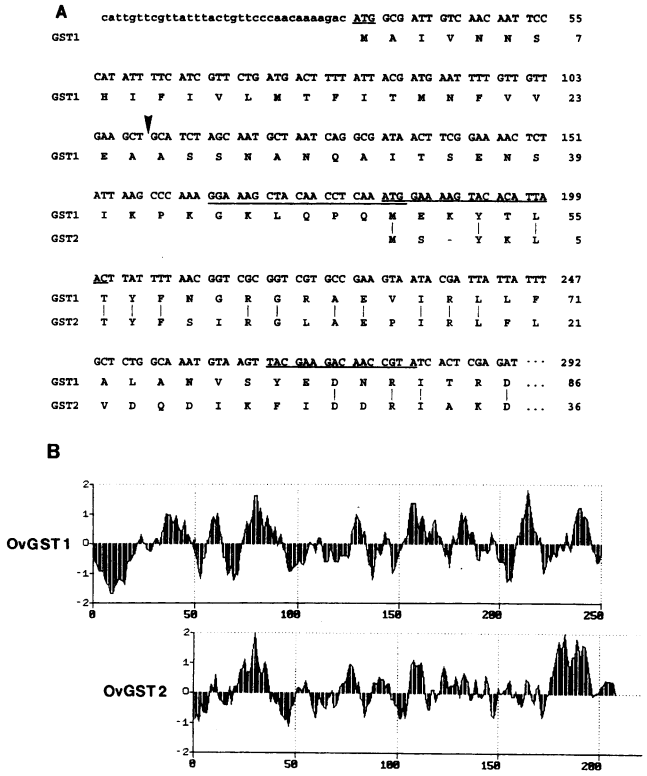

FIG. 1. (A) 5′ cDNA sequence amplified by the RACE procedure and the deduced N-terminal amino acid sequence of OvGST1 up to residue 86 in comparison with the amino acid sequence of the second *O. volvulus* GST isolated in our laboratory (OvGST2) (21). The putative initiation codon is underlined. The arrowhead marks the cleavage site and the start of the mature protein. The nucleotide sequence corresponding to the primers used in the RACE procedure are underlined. (B) Hydrophobicity plot of the OvGST1 and OvGST2 protein sequences averaged over 10 residues. Number on the horizontal axis indicates amino acid residue. Hydrophobicity is demonstrated by increasing negative numbers on the vertical axis, while hydrophilicity is shown by increasing positive numbers.

between the h-region and the cleavage site (c-region). The last residue before the cleavage site is an alanine, which is one of the amino acids commonly found in this position in other signal peptides (31). Overall, 68% of these residues are hydrophobic, 24% are polar, and only 4% are negatively and 4% are positively charged. OvGST1 also possesses a 24-amino-acid stretch prior to the homology to other known GSTs (Fig. 1A). In this region, 50% of the residues are polar, 33% are hydrophobic, 4% are negatively charged, and 13% are positively charged. The positively charged residues (lysines) are at the carboxy terminus at positions 41, 43, and 45. In the same region, at positions 42, 48, and 44, there are amino acids with alpha-helix-breaking properties (two prolines and one glycine, respectively). A specific function of this region remains to be determined.

In vitro secretion. Live female worms were maintained in culture medium; the medium was collected and concentrated, separated by SDS-PAGE, and blotted onto nitrocellulose filters which were probed with anti-rOvGST1 serum. The viability of the worms was examined by measuring the production of lactate over an extended period, as previously described (16). Figure 2 shows the identification of the 31-kDa OvGST1 in spent medium (lane 2). The additional band at 34 kDa (lane 2) corresponds to a protein which is isolated from *O. volvulus* extract along with the described OvGST1 when glutathione affinity chromatography is used. Neither protein is observed in the control medium (lane 1). The N-terminal sequence of the 34-kDa protein has been determined (unpublished data) and found to be very homologous to that of OvGST1, varying in only 3 of 17 amino acid positions. It is not the previously described OvGST2 (21), which is 24 kDa and possesses a distinct amino acid sequence. It is either a counterpart of OvGST1 with amino acid variation due to the pooled worm population used in the preparation or another isoform of OvGST. The cross-reactivity of the anti-rOvGST1 serum with the 34-kDa protein also indicates that it must have structural similarities with the OvGST1.

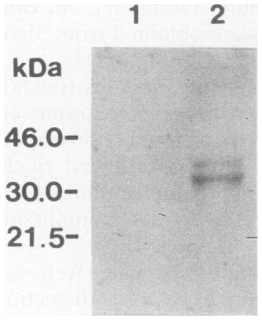

FIG. 2. Identification of OvGST1 in spent medium of *O. volvulus*. Lane 1, control culture medium (no worms); lane 2, spent medium. Identification of OvGST1 was done by Western blot with specific anti-rOvGST1 serum.

Glycosylation of OvGST1. A wide variety of eukaryotic membrane-bound and secreted proteins possess covalently bound carbohydrates. Posttranslational modification of the polypeptide OvGST1, in the form of N-glycosylation, was investigated because of the 4-kDa size difference between the native mature enzyme (without a signal peptide) and recombinant enzyme encoded by the cDNA (without a signal peptide), which were expected to be equivalent. The OvGST1 amino acid sequence possesses four potential N-glycosylation sites (20). Here, the presence of N-linked oligosaccharides on OvGST1 was shown by the sensitivity of OvGST1 to the enzyme *N*-glycosidase F (*N*-glycanase F), which cleaves the *N*-glycan linkage of glycoproteins between asparagine and the carbohydrate chain. Figure 3 shows a Western blot in which the native OvGST1 was detected in *O. volvulus* extract with anti-rOvGST1 serum before (lane 1) and after (lanes 2 to 6) cleavage of oligosaccharides by different concentrations of the enzyme *N*-glycosidase F. To aid in the size comparison, rOvGST1, raised in *E. coli* (20), was also applied (lane 7). Figure 3 shows that, in a concentration-dependent manner, the *N*-glycanase F cleaves two oligosaccharide chains stepwise, resulting in a deglycosylated native OvGST1 polypeptide of approximately the same size as the nonglycosylated rOvGST1 (27 kDa), which was designed to start at the N terminus of the native OvGST1. As previously described (20), the smaller 24-kDa protein observed in lane 7 is due to translation initiation in *E. coli* at an internal methionine in the primary sequence, at position 50, which was confirmed by N-terminal protein sequencing of both of the *E. coli*-produced proteins.

In this experiment, as in the detection of OvGST1 in spent media, the anti-rOvGST1 serum detects two distinct proteins in the *O. volvulus* 100,000 × *g* supernatant before treatment with *N*-glycanase (lane 1). After treatment, the 34-kDa protein

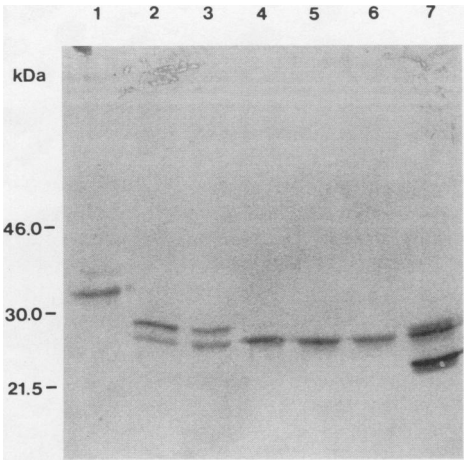

FIG. 3. Cleavage of oligosaccharide side chains in *O. volvulus* 100,000 × *g* supernatant after 12 h of incubation with *N*-glycanase F. Lane 1, no enzyme; lane 2, 1 mU; lane 3, 10 mU; lane 4, 100 mU; lane 5, 1 U; lane 6, 5 U; lane 7, rOvGST1. Identification of OvGST1 was done with specific anti-rOvGST1 serum.

and the 31-kDa OvGST1 are reduced to a polypeptide backbone of the same size, since the 34-kDa band disappears completely and no new, unidentified bands of another size appear. It is likely that these two proteins are different glycoforms (26) of the same type of GST, but this remains to be determined. Another possibility is that during the preparation of the parasite one of the oligosaccharide chains of OvGST1 is not retained.

Immunolocalization. The anti-OvGST1 stained discrete portions of adult worms, while preimmune serum showed minimal or no staining (Fig. 4A and C). Satisfying labelling results for OvGST1 were achieved at a dilution of 1:1,000; therefore, the following results are based on experiments using this dilution. With few exceptions, mainly structures of the midbody region of mature females are described.

The strongest immunostaining for OvGST1 was observed in the outer cellular covering of the body, the syncytial hypodermis, mostly in the interchordal hypodermis in most worms examined (Fig. 4B). The peripheral membrane forms series of lamellae, which run in the outer zone of the hypodermal cytoplasm. This is where the most intense immunostaining for OvGST1 was consistently observed (Fig. 4D). The thick lateral, dorsal, and ventral hypodermal chords showed in most cases only positive immunolabelling for OvGST1 in their outer zones (Fig. 4B).

In every worm examined, there was no immunostaining for OvGST1 in the longitudinal musculature (Fig. 4D). In the epithelial cells of the intestinal wall, as well as in the uterine wall, no overall labelling was observed, except that in a few worms there was a weak immunostaining for OvGST1 in the uterine epithelial cells. Occasionally, some embryonic stages with weak to moderate immunostaining for OvGST1 were observed in the uterus.

Coiled microfilariae surrounded by labelled eggshells showed some labelling, but in most cases all embryonic stages were completely unlabelled. Outside the adult females microfilariae with no distinct immunostaining were observed.

In less than 10% of the worms examined, immunodetection of GST on the outside of the cuticle as well as a moderate labelling of adjacent multinucleated giant cells or macrophages were observed.

Gel filtration. The molecular weight of the enzymatically active rOvGST1 was estimated to be approximately 50,000 to 60,000. This estimate is close to the sum of two subunits, and it was concluded that the active OvGST1 is dimeric.

DISCUSSION

The results presented in this report provide evidence that OvGST1 appears to be peripherally associated with the outer membrane of the hypodermis. Proteins that are secreted from the cell are first sequestered across the endoplasmic reticulum by virtue of a cleavable NH_2-terminal signal sequence and are then subject to cotranslational modifications such as N-glycosylation. The new OvGST1 sequence data obtained by the RACE method revealed that the 25 N-terminal amino acids possess the characteristics of a signal peptide sequence. The cleavage site also fits the (−3, −1) rule (33), which was confirmed experimentally by N-terminal amino acid sequencing of the native OvGST1 purified from adult worms. The mature OvGST1 is lacking the signal peptide encoded by the cDNA. It was also demonstrated that the native OvGST1 is glycosylated.

The data presented here are, to our knowledge, the first to describe a glycosylated GST possessing a signal peptide sequence belonging to the "hydrophobic" group, consisting of a tripartite structure that directs proteins across the endoplasmic reticulum membrane. The human and rat mitochondrial matrix GSTs (class theta), of course, have a short terminal presequence that is not present in the mature form of the enzyme, but this signal sequence displays residues which are conserved among mitochondrial import presequences (14). Sm28 GST has a potential signal peptide, but this is not processed, as was shown by N-terminal peptide sequencing of the native protein (4). The only two microsomal GST sequences found so far appear to possess no cleavable signal sequences (11).

When other GSTs which are glycosylated have been assessed, there has been evidence in one study (18) of a glycoconjugate form of rat GST Yp and human GST pi, but no signal peptide is apparent in either form. Furthermore, another study about isotype responses to candidate vaccine antigens in sera obtained from mice vaccinated with irradiated cercariae of *S. mansoni* (27) reports that GST was predominantly recognized by immunoglobulin M antibodies of all vaccinated groups and that about half of the immunoglobulin M antibodies were directed against carbohydrate epitopes.

The signal peptide is followed by a stretch of 24 amino acids, which are not present in other GSTs described to date. Speculations on the function of this novel portion of the polypeptide include effects on enzyme activity or substrate specificity. The three positively charged residues and the three amino acids with alpha-helix-breaking capacity, all at the carboxy end of the extension, perhaps constitute a structural motif that is important for the localization of OvGST1.

We are currently testing the substrate specificity of rOvGST1 expressed in *E. coli* with and without the N-terminal extension (unpublished data). The results to date strongly indicate the need to express OvGST1 in a eukaryotic expression system. OvGST1 differs not only in having the N-terminal extension but also in that it is glycosylated. Eukaryotic protein modifications may contribute to the physiological activity or modulate the physiocochemical properties of the protein.

The four different classes of cytosolic GSTs usually function as dimeric proteins that are assembled from identical or nonidentical subunits. As previously described, OvGST1 has 22.9% sequence identity to rat GST of the pi class (20). This

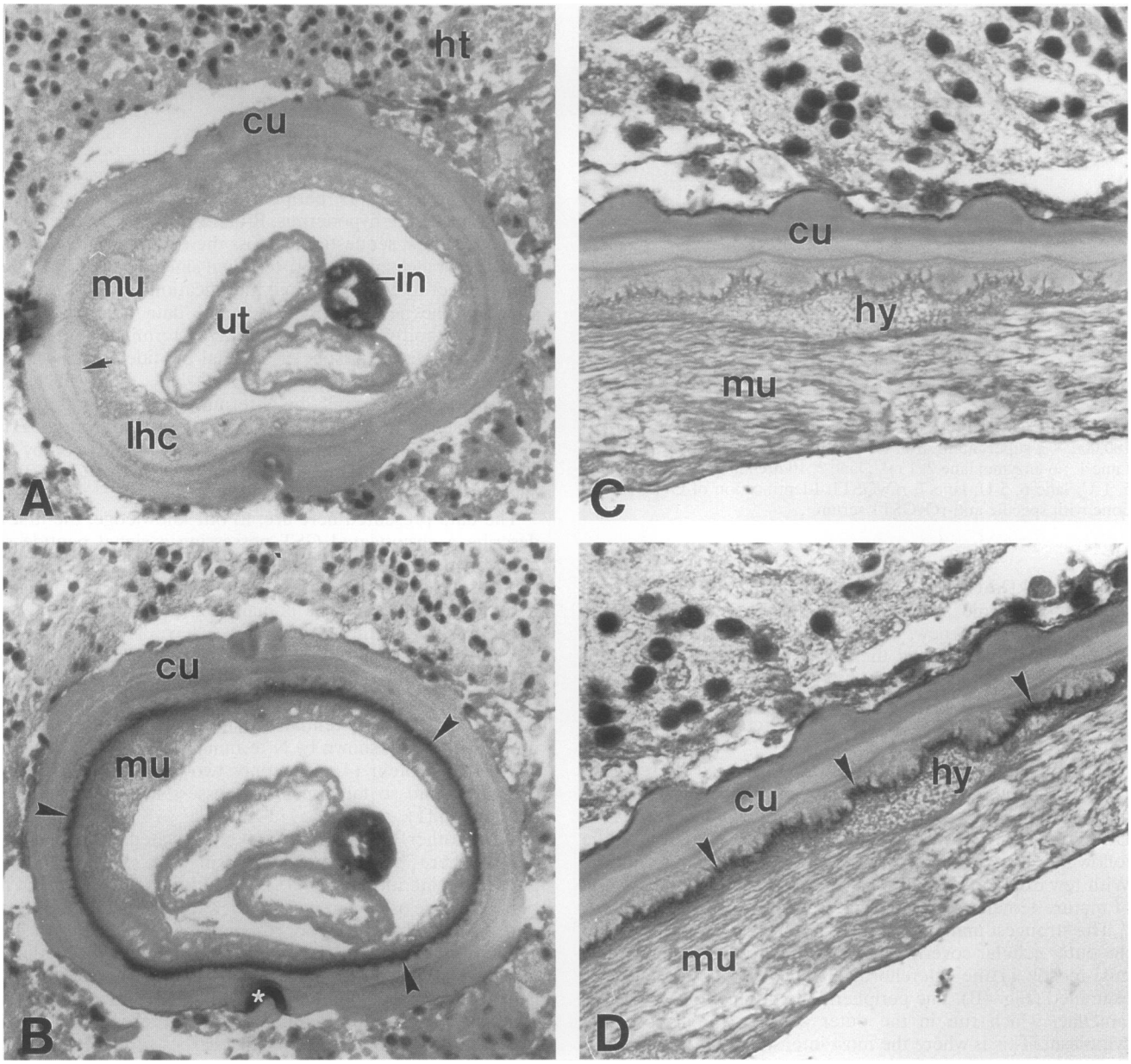

FIG. 4. (A and B) Cross section of a middle-aged female *O. volvulus* worm with empty uteri. (A) Immunostained with the preimmune serum. No labelling was observed in the hypodermis (arrow) or any other part of the worm. (B) Immunostained with anti-rOvGST1. Intense labelling was observed in the interchordal hypodermis (arrowheads). The hypodermal cords showed no labelling. Magnification, ×160. (C and D) Longitudinal section of the body wall of a mature female worm showing normal ridges of the cuticle, which is covered by the surface coat. (C) Immunostained with preimmune serum. (D) Immunostained with anti-rOvGST1. Intense labelling was achieved in the outer zone of the hypodermis. Magnification, ×670. The arrowheads indicate immunostaining in the hypodermis. cu, cuticle; ht, host tissue; hy, hypodermis; in, intestine; lhc, lateral hypodermal cord; mu, muscle cells; ut, uterus. *, cuticular fold (preparational artifact).

coincides with the result that OvGST1 appears to be active as a dimer, as is the case with the pi class GSTs (12).

To aid in assessing the role of this novel GST in *O. volvulus*, we localized OvGST1 in *O. volvulus* female and male adult worms by immunodetection at the light-microscopic level. The staining properties of the polyclonal antibody OvGST1 were affected by the type and duration of fixation. The unique localization profile observed may indicate that OvGST1 has a parasite-specific function. The outer portion of the hypodermis, which might facilitate the exchange of material with the cuticle and environment (13), was the most intensely labelled part of the worm. Mitochondria are distributed throughout the hypodermis, but in close association with the outer membrane there is a particular zone in which many mitochondria are found beside lysosome-like bodies and multivesicular bodies. Especially in female worms, this zone is probably responsible for the metabolism of materials which are incorporated from the environment and also for those materials which are excreted or secreted to the cuticle and to the environment (13).

When considering the detoxifying function of the GSTs and

the results obtained from immunolocalization, one could speculate that OvGST1 might be closely associated with the lysosome-like bodies or the multivesicular bodies which are numerous in the outer hypodermis. The in vitro experiment shows OvGST1 to be released from adult worms into the culture medium, but whether this mimics the situation in vivo is not clear. The localization and route of release of the OvGST1 will be further investigated by immunoelectron microscopy.

The results presented here strongly suggest that OvGST1 may be involved in protecting the parasite near the host-parasite interface.

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