

## Research Article

# Release of Glycoprotein (GP1) from the Tegumental Surface of *Taenia solium* by Phospholipase C from *Clostridium perfringens* Suggests a Novel Protein-Anchor to Membranes

Abraham Landa,<sup>1</sup> Kaethe Willms,<sup>1</sup> and Juan Pedro Laclette<sup>2</sup>

<sup>1</sup>Departamento de Microbiología y Parasitología, Facultad de Medicina, UNAM, México 04510, DF, Mexico

<sup>2</sup>Departamento de Inmunología, Instituto de Investigaciones Biomédicas, UNAM, México 04510, DF, Mexico

Correspondence should be addressed to Abraham Landa, landap@servidor.unam.mx

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In order to explore how molecules are linked to the membrane surface in larval *Taenia solium*, whole cysticerci were incubated in the presence of phospholipase C from *Clostridium perfringens* (PLC). Released material was collected and analyzed in polyacrylamide gels with sodium dodecyl sulfate. Two major bands with apparent molecular weights of 180 and 43 kDa were observed. Western blot of released material and localization assays in cysticerci tissue sections using antibodies against five known surface glycoproteins of *T. solium* cysticerci indicated that only one, previously called GP1, was released. Similar localization studies using the lectins wheat-germ-agglutinin and Concanavalin A showed that N-acetyl-D-glucosamine, N-acetylneuraminic, sialic acid, α-methyl-D-mannoside, D-mannose/glucose, and N-acetyl-D-glucosamine residues are abundantly present on the surface. On the other hand, we find that treatment with PLC releases molecules from the surface; they do not reveal Cross Reacting Determinant (CRD), suggesting a novel anchor to the membrane for the glycoprotein GP1.

## 1. Introduction

The tegumental surface of larval cestodes is in direct contact with the host tissues and plays a crucial role in the survival of the parasite. Glycoproteins and complex carbohydrates have been detected on the surface of the larvae in several species of cestodes through the use of histochemical techniques [1–4].

The tegumental membrane of *Taenia solium* cysticerci exhibits a dense glycocalyx composed of abundant carbohydrates and glycoproteins such as GP1, GP2-3, GP6, and GP7 [5–7]. However, little is known about the anchorage of the glycoproteins and glycolipids to the membrane. In other platyhelminths, several studies have also shown that alkaline phosphatase, acetyl-cholinesterase, and several surface proteins of 18, 22, 28, 32, 38, and 200 kDa are anchored to the tegumental membrane via glycosyl-phosphatidyl-inositol (GPI) in adult and somules of *Schistosoma mansoni* [8–10]. Moreover, the apical gut surface protein (p46<sup>Gal</sup>) of *Haemonchus contortus* is also anchored through GPI [11].

Likewise, Sm25, a major schistosome tegumental glycoprotein, is attached by palmitic acid to the membrane [12].

Cestodes, trematodes, and other platyhelminths have triglycerides and cholesterol as the major neutral lipids and phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine as the major phospholipids [13, 14]. Glycolipids, galactosylceramides, and glycosphingolipids have been identified in tegumental membranes of *Spirometra mansonioides*, *Echinococcus multilocularis*, and *Hymenolepis diminuta* [15–17]. A novel glycosphingolipid named AGL containing inositol phosphate as acidic group has been found in the nematode *Ascaris sum* [18].

The purpose of this study was to determine the components that are released from the surface of *T. solium* cysticerci by phospholipase C from *Clostridium perfringens* (PLCs). The GP1 molecule was removed by PLC. Finally, complex carbohydrates that are ligands for wheat germ agglutinin (WGA) and concanavalin A (ConA) were also released from the tegumental surface. These results suggest that a different kind of glycoprotein anchor might be present in taeniids.

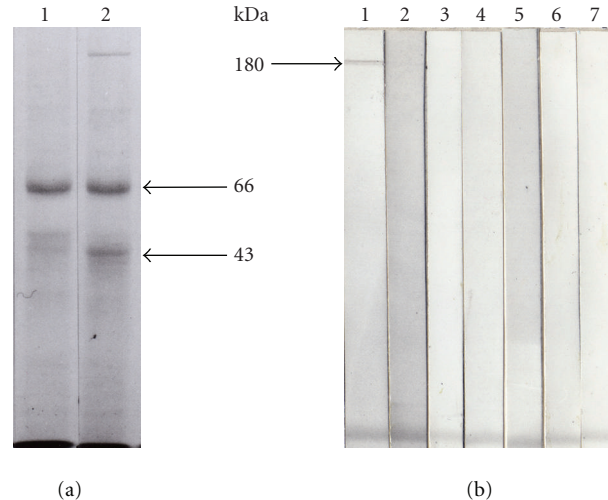


FIGURE 1: Materials released by PLC from the surface of *T. solium* cysticerci. (a) SDS-PAGE showing the material released after 1 hour incubation in Lane 1: PBS and Lane 2: PBS-PLC. (b) Western Blot of the material released with PLC probed with IgG fraction of Lane 1: anti-GP1, Lane 2: anti-GP2-3, Lane 3: anti-GP6, Lane 4: anti-GP7, Lane 5: anti-pig IgG, Lane 6: Preimmune rabbit serum, and Lane 7: anti-cross reacting determinant (CRD).

## 2. Materials and Methods

**2.1. Biological Material.** *Taenia solium* cysticerci were dissected from skeletal muscle of naturally infected pigs obtained in local abattoirs. Cysticerci were immediately washed three times with sterile phosphate buffered saline, pH 7.2 (PBS), containing 100  $\mu\text{g}/\text{mL}$  of penicillin and 100 U/mL of streptomycin. Crude extract of *T. solium* was obtained as described before [19].

**2.2. Phospholipase C Treatment of Intact Cysticerci.** Two groups of 20 cysticerci each were incubated for 1 hour at room temperature with PBS or PBS containing 10 U/mL of phospholipase C (PLC), type XIV, from *C. perfringens* (Sigma Chemical Co.) in the presence of proteinase inhibitors (0.1 mg/mL aprotinin, 5 mM PMSF, and 2.5 mM TLCK). Supernatants were collected and the amount of protein released was determined in each fraction with the Bio-Rad Protein Assay (Bio-Rad Laboratories, California, USA).

**2.3. Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis.** Samples of 10  $\mu\text{g}$  of the released material were mixed with sample buffer (3% SDS, 62.5 mM Tris-HCl and 5% 2-mercaptoethanol, pH 6.8) and boiled for 2 minutes. Samples were run on 10% polyacrylamide gels and stained with Coomassie Blue R-250. Protein bands were transferred onto nitrocellulose membranes (Millipore, Illinois, USA) and incubated for 1 hour at 37°C with the appropriate dilution of the IgG fraction of one of the following antisera:  $\alpha\text{GP1}$ ,  $\alpha\text{GP2-3}$ ,  $\alpha\text{GP6}$ ,  $\alpha\text{GP7}$ ,  $\alpha\text{pig IgG}$ , and anti-CRD of GPI. The IgG fraction of preimmune rabbit serum was used as negative control. Peroxidase-conjugated goat anti-rabbit IgG (Zymed) was used as secondary antibody. Bound antibodies were developed incubating the blots with

0.5 mg 3-3'-diaminobenzidine and 20  $\mu\text{L}$  of 1%  $\text{H}_2\text{O}_2$  in PBS for 15 minutes at room temperature [20].

**2.4. Histochemical Studies.** Cysticerci that were treated with and without PLC were washed three times with PBS and fixed as described before [21]. Fragments of the bladder wall of cysticerci treated with PBS or PBS-PLC were incubated for 1 hour at room temperature with the appropriate dilution of the IgG fraction of one of the following antisera:  $\alpha\text{GP1}$ ,  $\alpha\text{GP2-3}$ ,  $\alpha\text{GP6}$ ,  $\alpha\text{GP7}$ , and  $\alpha\text{pig IgG}$ . The IgG fraction of a preimmune rabbit serum was used as negative control. Peroxidase-conjugated goat anti-rabbit IgG (Zymed) was used as secondary antibody. The bound antibodies were developed incubating the fragments with 0.5 mg 3-3'-diaminobenzidine and 20  $\mu\text{L}$  of 1%  $\text{H}_2\text{O}_2$  in PBS, for 15 minutes at room temperature. Ruthenium red (RR) was also used in the fixation of intact cysticerci [22], before and after PLC treatment following the procedure described before. Similar localization studies were carried out using horseradish peroxidase (HRP) conjugated to ConA or WGA. Peroxidase reaction was stopped in cold PBS and all cyst fragments were postfixated in osmium tetroxide and processed for light and electron microscopy as described before [19]. Semithin (2  $\mu\text{m}$ ) and thin (80 nm) sections with or without staining were photographed in a Nikon Optiphot microscope and a JEOL-1200 EXII electron microscope.

## 3. Results

Treatment of the cysticerci with PBS and PBS-PLC resulted in the release of several proteins (Figure 1(a)). The supernatant from the cysticerci treated with PBS alone (lane 1) shows one intense band with an apparent molecular weight of 66 kDa and several weak bands around 90, 50, 45, 30, and 15 kDa. In contrast, treatment with PBS-PLC resulted in a similar

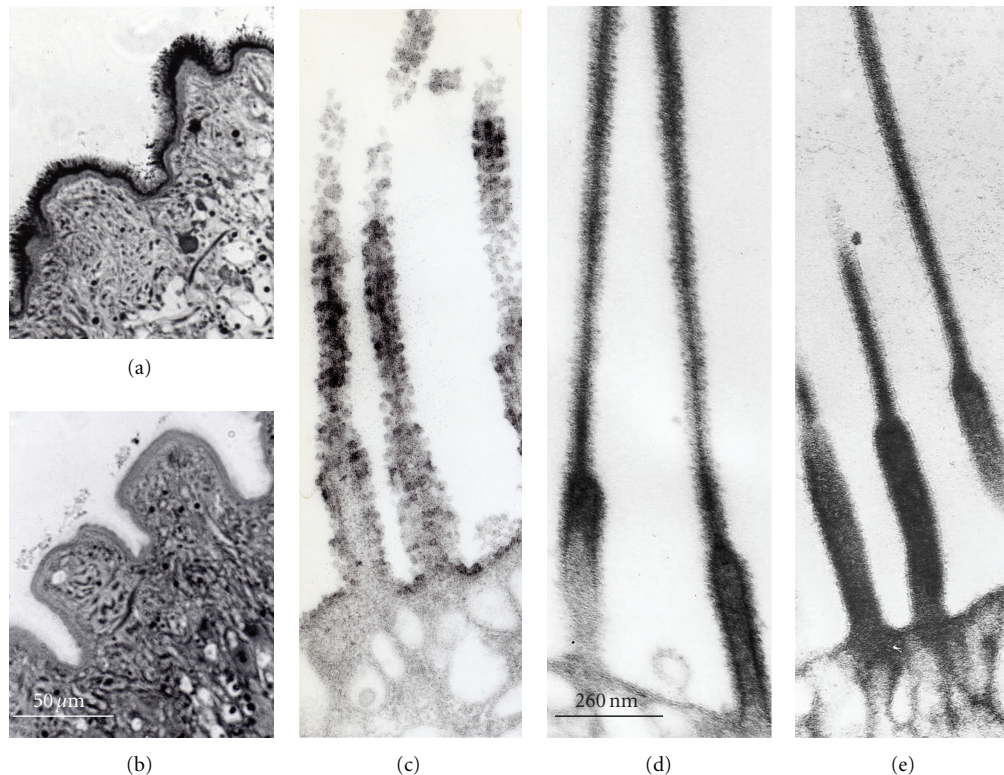


FIGURE 2: Micrographs of the tegumentary surface of *Taenia solium* cysticerci: light and electron micrographs of sections of the bladder wall of cysticerci incubated for 1 hour with PBS ((a) and (c)) and PLC ((b) and (d)) probed with anti-GP1. (e) IgG from preimmune serum rabbit was used as control. HRP-conjugated goat anti-rabbit IgG was used as secondary antibody.

band pattern as well as three major bands of 180 kDa, 66, and 43 kDa and a light smear under 45 kDa (Lane 2). Western blot assays using different antibodies against previously described tegumental glycoproteins  $\alpha$ GP1,  $\alpha$ GP2-3,  $\alpha$ GP6, and  $\alpha$ GP7, as well as against  $\alpha$ pig IgG, showed that the only protein released by the PBS-PLC treatment was recognized by  $\alpha$ GP1 antibodies, with an apparent molecular weight of 180 kDa (Figure 1(b)). The molecules contained in the PBS-PLC fraction were negative to the anti-CRD antibodies. Exposure of CRD requires cleavage by phosphatidylinositol-specific phospholipase C (PIPLC) of GPI at the junction between the phosphate and the hydrophobic diacylglycerol moiety [23, 24].

Localization studies with light and electron microscopy were carried out to determine if changes on the surface glycoprotein pattern could be observed after PBS-PLC treatment. As shown in the light and electron micrographs in Figures 2(a) and 2(c), a positive peroxidase reaction is present on the tegumentary and microtriche surface with anti-GP1 antibodies, but the same antibodies did not recognize it on cysticerci after PLC treatment (Figures 2(b) and 2(d)), illustrating that GP1 is almost completely removed from the tegumental surface. In contrast, the molecules GP2-3, GP6, GP7, and pig IgG were still bound to the microtriche surface after treatment with PLC (Figures 3(a)–3(d)). For these experiments preimmune rabbit IgG was used as negative control (Figure 2(e)).

To ascertain that the PLC treatment induced changes in the general pattern of surface carbohydrates on cysticerci, RR and lectin staining were carried out. The PBS-PLC treatment of cysts did not induce a noticeable change on regular distribution of RR stained particles on the surface of microtriches (Figures 4(a) and 4(b)). Images obtained after exposure to ConA and WGA normally appear as electron dense particles on the outer surface of microtriches (Figures 4(c), 5(a), and 5(c)). Particles that bind ConA were less abundant after PBS-PLC treatment (Figure 4(d)). In contrast, PBS-PLC treatment abolished almost completely the binding of WGA as illustrated by the negative peroxidase reaction in Figures 5(b) and 5(d). Interestingly, the patches recognized by WGA were destroyed by the PBS-PLC treatment whereas the patches for Con A were preserved.

#### 4. Discussion

We have examined the effect of PLC treatment on plasma membrane lipids in *T. solium* cysticerci. PLC preferentially hydrolyses phosphatidylcholine but has a broad specificity for other lipids such as sphingomyelin, phosphatidylserine, and lysophosphatidylcholine, but not for glycosylphosphatidylinositol [25]. We have already demonstrated that glycoproteins GP1, GP2-3, GP6, and GP7 and pig IgG are abundant molecules seen as electron dense particles on the external surface of the tegument of *T. solium* cysticerci. GP1

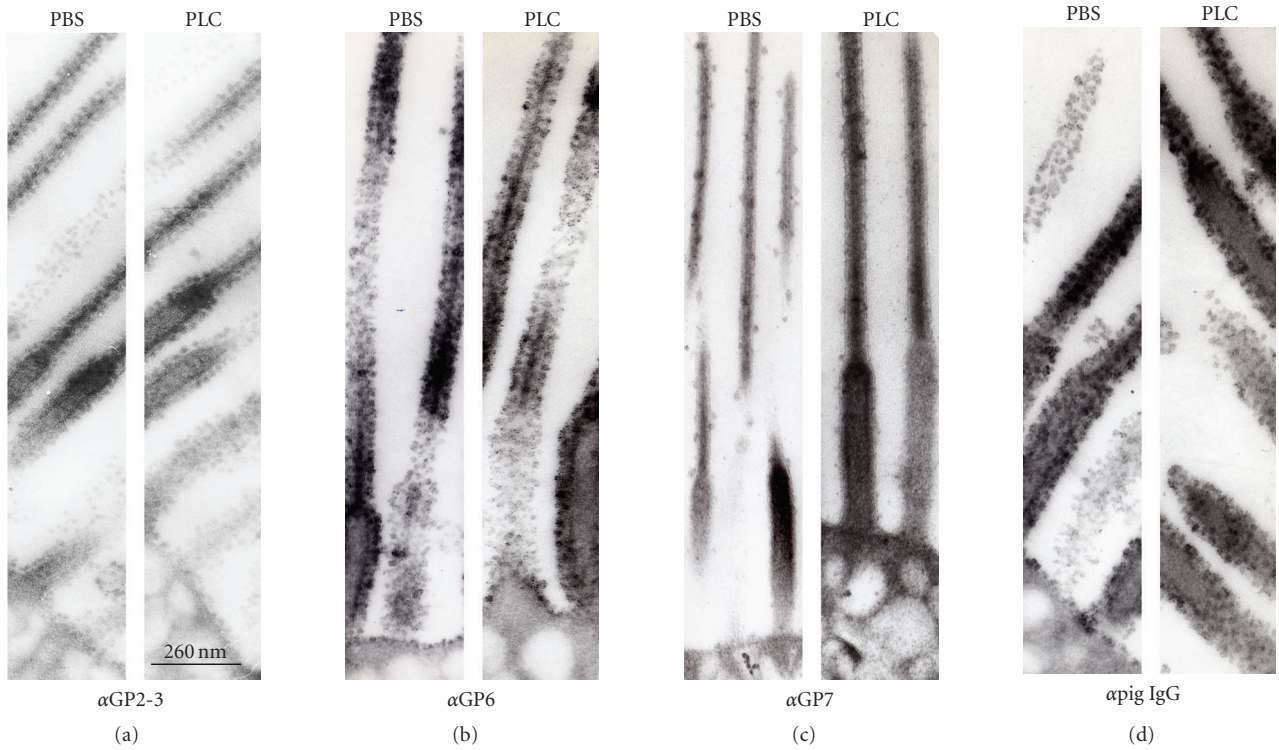


FIGURE 3: Tegument surface of *Taenia solium* cysticerci, after 1 hour incubation with PBS or PLC. Sections of the bladder wall of cysticerci were incubated with the IgG fraction of (a) anti-GP2-3, (b) anti-GP6, (c) anti-GP7, and (d) anti-pig IgG.

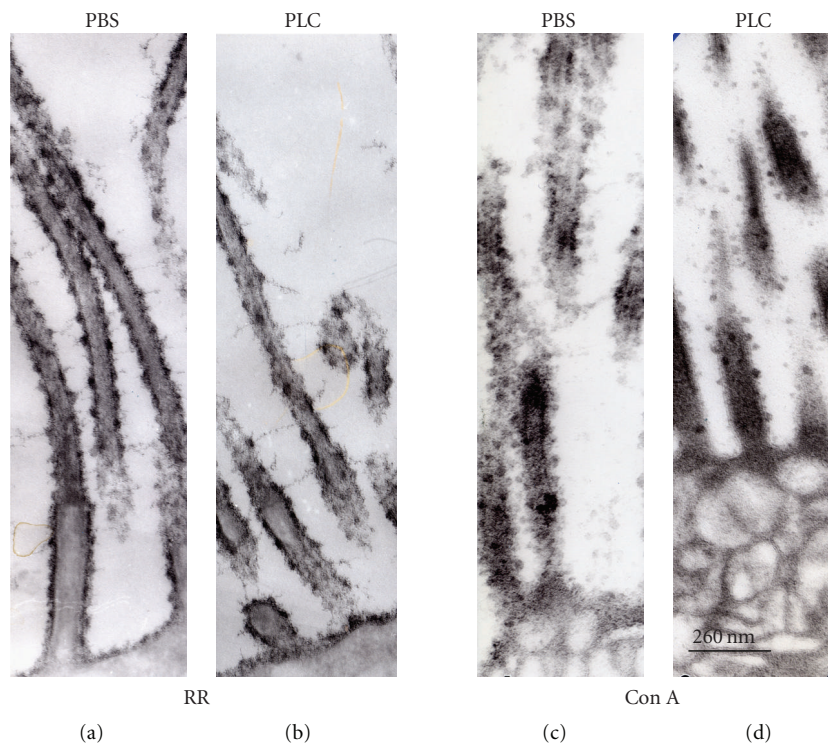


FIGURE 4: Electron micrographs of the tegumentary microtriches of *Taenia solium* cysticerci, after 1 hour incubation with PBS and PLC. Sections of the bladder wall were (a) and (b) stained with ruthenium red (RR) and (c) and (d) incubated with concanavalin A (ConA)-peroxidase.

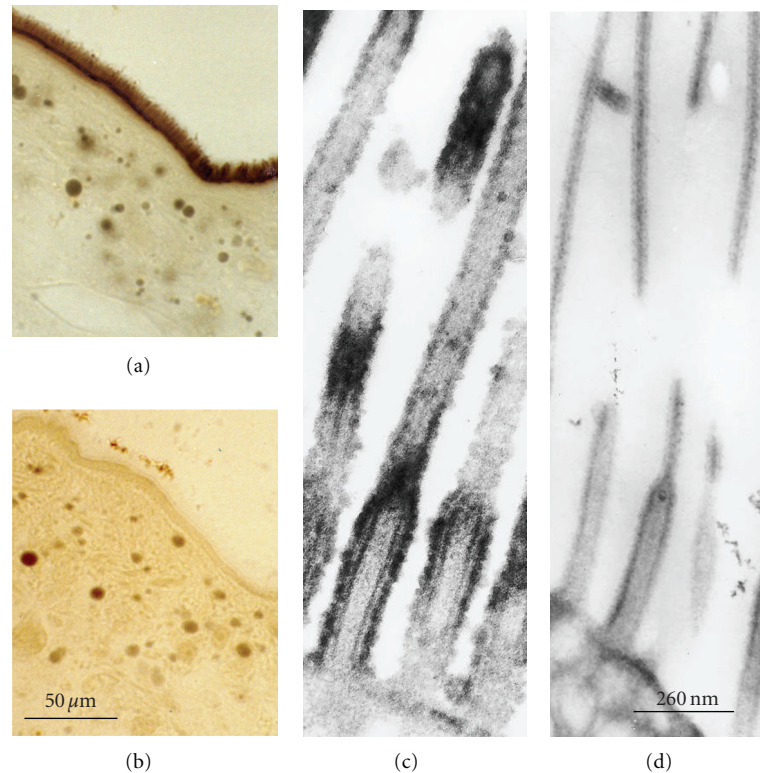


FIGURE 5: Light and electron micrographs of sections of the bladder wall of cysticerci incubated for 1 hour with PBS ((a) and (c)) and PLC ((b) and (d)) and probed with WGA-peroxidase.

is also present on the surface of *T. solium* and *T. saginata* adult worms as well as in *T. saginata*, *T. pisiformis*, and *T. crassiceps* cysticerci [7].

Western blot results showed that GP1 was present in the PLC released material, whereas CRD was absent (Figure 1). Moreover, light and electron microscopy images showed the loss of GP1 from the surface of cysticerci after treatment with PLC (Figure 2). All assays to determine the lipolytic activity of PLC were carried out in the presence of proteinase inhibitors that inhibit the effect of proteases in our preparation but do not affect the activity of PLC. In addition, data showed that the receptors to IgG and the other glycoproteins also present on the surface of cysticerci were not affected by PLC. This suggested that GP1 association with the tegumental membrane depends on an interaction with the phospholipids mentioned before. It is worth mentioning that PLC has an MW of 43 kDa and it is therefore possible that the band of around 43 kDa found in the PLC-released material could correspond to this enzyme used in the assay.

On the other hand, analysis of the general changes in the surface carbohydrates of *T. solium* cysts using ruthenium red and the lectins WGA and Con A suggested that the action of PLC is specific for a few surface components. The basic pattern of particles in the glycocalyx is clearly maintained after PLC treatment, as observed in the assays using RR and Con A. The removal of most of the binding sites for WGA suggested that N-acetyl-D-glucosamine, N-

acetylneuraminic, and sialic acid are important components of the released materials including the glycoprotein GP1. The ConA lectin principally binds to  $\alpha$ methyl-D-mannoside, D-mannose/glucose, and N-acetyl-D-glucosamine. The almost complete removal of binding sites to WGA and slight decrease in the binding sites for ConA after PLC treatment suggests that sugars recognized by both lectins are present on the surface. These findings are in agreement with results reported in other cestodes [1, 26]. It seems clear that the particles observed on the surface are complex structures composed of different molecules, among them, the sugars and glycoproteins.

It is known that products of the phospholipases such as phosphatidylinositol and diacylglycerol are recognized as important reservoirs of second messenger precursors and as anchors for membrane enzymes.

Our report is the first demonstration of a novel type of membrane anchor; however the biological role of these structures in *Taenia* genus remains to be explored.

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