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Cell Surface Glycoproteins in *Crithidia deanei*: Influence of the Endosymbiont

Benedito Prado DIAS FILHO, Tânia UEDA-NAKAMURA, Carlos Henrique LOPEZ[1], Luiza Tamie TSUNETO, Benício Alves ABREU FILHO and Celso Vataru NAKAMURA

Laboratório de Microbiologia, Departamento de Análises Clínicas, Universidade Estadual de Maringá, Maringá, Paraná, Brazil

Summary. *Crithidia deanei*, a protozoan of the family Trypanosomatidae harboring an endosymbiont bacterium in its cytoplasm and cured strain (endosymbiont-free) were compared as to glycoprotein composition. The wild strain of *C. deanei* showed a double band with molecular mass of 70/74 kDa. These bands were absent from symbiont-free cells. Indirect immunofluorescence microscopy using specific antibodies against the 70/74 kDa glycoprotein isolated from the symbiont-containing cells revealed intense labeling of the cell surface in the symbiont-harboring *C. deanei*. However, no such labeling was observed in symbiont-free cells. These observations suggest that the endosymbiont influences the composition of the glycoprotein on the cell surface of *C. deanei.*

Key words: *Crithidia deanei*, endosymbiont, glycoproteins, immunofluorescence.

INTRODUCTION

The large family Trypanosomatidae includes some species associated with human, animal, or plant diseases. Most of the studies involving this family have focused on the human-pathogenic species, and few biochemical analyses have been attempted on the so-called non-pathogenic trypanosomatids. Some trypanosomatids: *Crithidia deanei*, *C. oncopelti*, *C. desouzai*, *Blastocrithidia culicis*, and *Herpetomonas roitmani* harbor endosymbiotic bacteria (De Souza and Motta 1999). The ability of high doses of antibiotics to eliminate the endosymbiont has increased interest in the study of endosymbiont-harboring species, because several bacteria-protozoa interactions can be analyzed by comparing "cured" (endosymbiont-free) and wild strains. It is recognized that these intracellular symbionts are considerably integrated into the physiology of the host cell (McGhee and Cosgrove 1980). Furthermore, the presence of symbionts induces several morphological alterations in the host cells, such as the rearrangement of kinetoplast DNA fibers and the disappearance of the paraxial rod structure observed in *C. deanei*, *C. oncopelti*, and *Blastocrithidia culicis* (Freymüller and Camargo 1981). The endosymbiont causes addi-

Address for correspondence: Celso Vataru Nakamura, Universidade Estadual de Maringá, Av. Colombo, 5790, CEP 87020-900, Maringá, Paraná, Brazil; Fax: +55 (044) 261-4860; E-mail: cvnakamura@uem.br

[1]Present address: Universidade Paranaense, Praça Mascarenhas de Morais, s/n, Umuarama, Paraná, Brazil

tional modifications, such as: a different carbohydrate composition of glycocalyx in *C. fasciculata*, a reduced surface charge and influence on the heme-synthesis pathway in *C. deanei*, the urea cycle in *B. culicis*, the enzyme threonine deaminase, nutritional requirements in *B. culicis*, *C. oncopelti*, and *C. deanei*, and the secretion of proteinases in *C. desouzai*, *C. deanei*, and *C. oncopelti* (De Souza and Motta 1999; Esteves *et al.* 1982; Oda *et al.* 1984; d'Avila-Levy *et al.* 2001, 2003). For all these reasons, endosymbiont-bearing trypanosomatids constitute excellent models for studies on symbiosis, which may contribute to better understanding of the origin of organelles in eukaryotic cells.

Carbohydrates are only a minor fraction of cell components, but they play an important role in the regulation of cell growth, antigenicity, and cell recognition (Nicolson 1974, 1976). Most of the carbohydrates exposed on the cell surface are bound to proteins, and therefore many studies have focused on membrane glycoproteins. Previous studies have reported that trypanosomatid protozoans do not contain cytoplasmic storage polysaccharides, so that carbohydrates are associated with cell membranes (De Souza 1989). In general, *C. deanei* and other trypanosomatids such as species of *Trypanosoma*, *Leishmania*, and *Herpetomonas* show similar profiles of cell-surface carbohydrates (Dwyer 1977, Chiari *et al.* 1978, Sixel *et al.* 1978).

The present investigation demonstrated the influence of the endosymbiont on the composition of the glycoproteins present on the cell surface of *C. deanei* grown in a chemically defined medium, through the use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/indirect immunofluorescence assays.

MATERIALS AND METHODS

Microorganism. Cultures of symbiont-bearing *Crithidia deanei* (ATCC 30255) were maintained by weekly transfers into a chemically defined medium (Mundim *et al.* 1974), added in 5-ml volumes to screw-capped tubes. The symbiont-free strain of *C. deanei* was maintained in the same defined medium with 0.030 g/l of nicotinamide (Sigma Chemical Company, St. Louis, USA) (Mundim and Roitman 1977). Cells were grown at 28°C for 48 h and stored at 4°C.

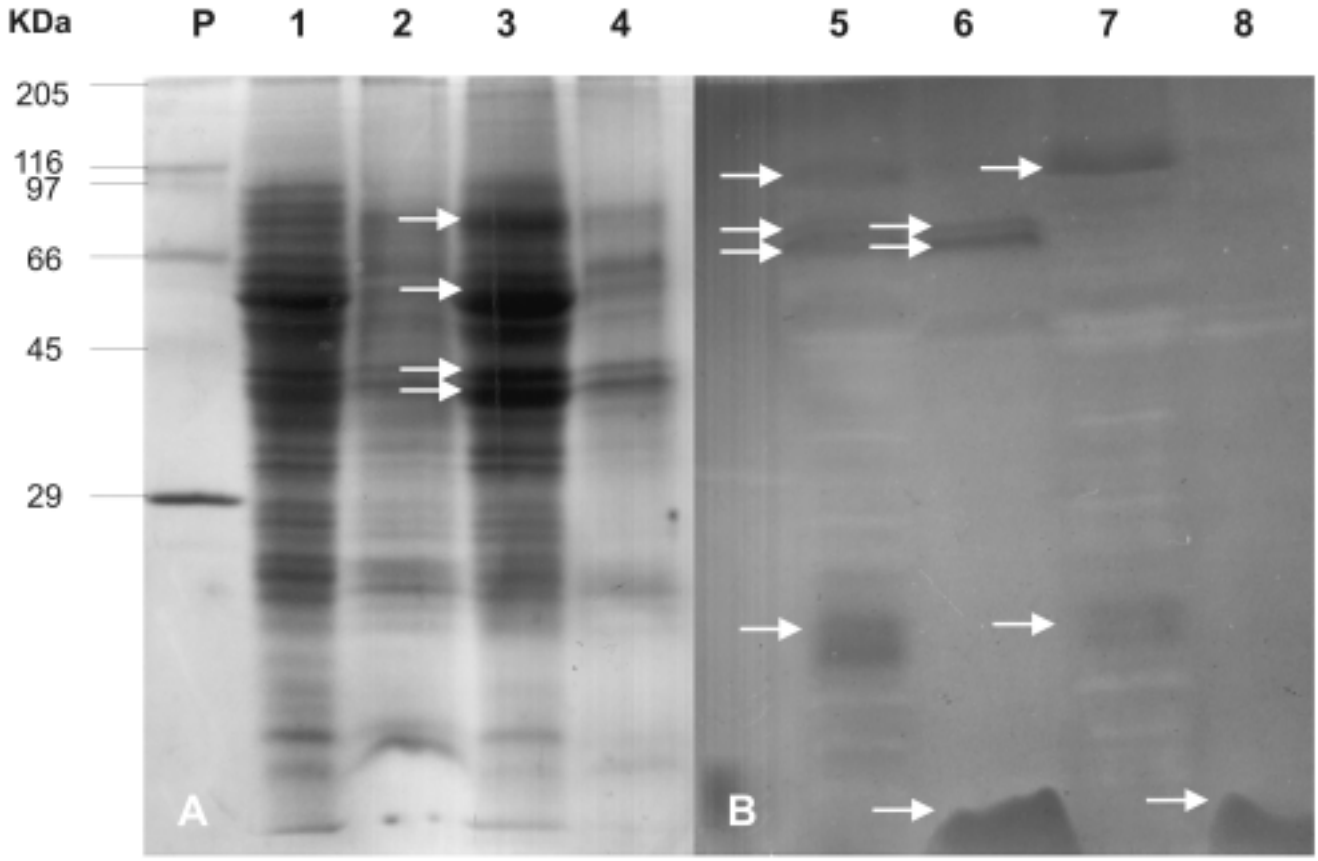
Cells cultivated in 1.5 l of defined medium (7×10^7 cells/ml) were harvested at the exponential phase (48 h) by centrifugation for 10 min at 2,000 g at 4°C and washed 4 times with cold 0.01M phosphate-buffered saline (PBS) pH 7.2.

Triton X-114 extraction. Symbiont-containing and symbiont-free cells (1×10^8 cells/ml) were solubilized in 2% Triton X-114 (Sigma) pre-condensed in Tris-saline buffer (TSB) (10 mM Tris - Invitrogen Life Technologies, 150 mM NaCl, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma) for 30-40 min at 0-4°C. Insoluble material was removed from the lysate by centrifugation at 20,000 g for 30 min at 0-4°C. The hydrophobic and hydrophilic phases were separated using a 6% (w/v) sucrose (Sigma) cushion (1:1.5, v/v) as described by Bordier (1981). Separation of the detergent from the proteins was effected by addition of zinc chloride (Mallinckrodt) to the samples, to obtain a final concentration of 0.05 M. The glycoproteins were precipitated by addition of 5 volumes of cold acetone (Merck). The pellet was collected by centrifugation, and the supernatant solution containing Triton X-114 was discarded. The proteins were resuspended in water and precipitated again with 5 volumes of cold acetone. After centrifugation, the pellet was washed with 50% cold acetone, dried at room temperature, and stored at -20°C until use.

SDS-PAGE electrophoresis. Samples of proteins and glycoproteins (hydrophobic and hydrophilic phases) precipitated with acetone were solubilized in the same volume of hot sample buffer (10 mM Tris-HCl, pH 8.0 1 mM EDTA, 1.0% SDS and 0.5% β-mercaptoethanol) and further boiled for 3 min. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (12% SDS-PAGE - Gibco Invitrogen Corporation, New York, USA) was run in duplicate (Laemmli 1970). Proteins were revealed by soaking in 0.25% (w/v) Coomassie Brilliant Blue R-250 (Sigma), in 50% (v/v) methanol and 10% (v/v) acetic acid. Destaining was achieved in the stain diluent. Glycoproteins were detected by staining the gel by the modified periodic acid-Schiff (PAS) method proposed by Doerner and White (1990). Briefly, gels were fixed overnight in 7.5% (v/v) acetic acid (Vetec) for 30 min, followed by addition of periodic acid (Sigma) for 1 h at 4°C and then incubated with Schiff's reagent for 1 h at 4°C. Reddish-pink bands of stained glycoprotein would then be visible. Reduction with 2% (w/v) sodium metabisulfite (Mallinckrodt) in 7.5% (v/v) acetic acid was performed overnight and subsequently stored in water.

Extraction and purification of 70/74 kDa glycoprotein. The cells were subjected to one cycle of freezing-thawing, cold water was added, and the mixture centrifuged at 3,000 g for 30 min. The resulting pellet was dispersed in cold water and the suspension once more centrifuged. The combined supernatants were heated at 100°C and lyophilized. The pellet remaining after centrifugation was extracted with aqueous phenol at 75°C (Mendonça-Previato *et al.* 1983), the aqueous layer dialyzed 3 times against 0.05 M phosphate buffer, pH 7.0, for 48 h, and applied to a Sephadex G-100 (Pharmacia Biotech) equilibrated column and run (1 ml/min) with a 0.1 M phosphate buffer, pH 7.0, at 4°C. The column was eluted with the same buffer at a flow rate of 1 ml/min, and 1 ml fractions were collected. The 70/74 kDa glycoprotein from endosymbiont-containing *C. deanei* was obtained. The elution profile was calibrated with known standards (Blue-dextran = 2,000,000 Da, Yellow-dextran = 2,000 Da, and Vitamin B_{12} = 125 Da) (Amersham Biosciences).

Production and purification of antibody. Approximately 2 mg/ml of 70/74 kDa glycoprotein was mixed with an equal volume of complete Freund's adjuvant for the first injection, and with incomplete Freund's adjuvant for the subsequent injections. A rabbit was immunized once a week for six weeks, and once a month thereafter. Pooled sera from four different bleeds were used in the experiments (Dias Filho *et al.* 1999). Purification of the crude antibody was affected by adjusting to pH 8.0 by adding 1/10 volume of 1.0 M Tris (pH 8.0), and then passing through a Protein A bed column (Amersham Biosciences). After the bed had been washed with 10 column volumes



Figs 1 A, B. Triton X-114 fractionation of endosymbiont-containing and endosymbiont-free *Crithidia deanei* analyzed by SDS-PAGE stained by Coomassie brilliant blue R-250 (**A**) and stained by periodic acid-Schiff (PAS) (**B**). **Lanes 1, 2, 5, and 6** endosymbiont-harboring *C. deanei*, **Lanes 3, 4, 7, and 8** aposymbiotic strain of *C. deanei*, **Lane P** molecular mass markers, **Lanes 1, 3, 5, and 7** hydrophilic phase, **Lanes 2, 4, 6, and 8** hydrophobic phase, arrows major bands as described in Results. Amount of protein loaded on the gel = 30 µg in each slot.

of 100 mM Tris (pH 8.0) and 10 column volumes of 10 mM Tris (pH 8.0), the column was eluted with 100 mM glycine (Sigma) (pH 3.0). The eluate was collected in 1.5-ml conical tubes containing 50 µl of 1 M Tris (pH 8.0). The tubes were then mixed gently to return the pH to neutral. The immunoglobulin-containing fractions were identified by absorbance at 280 nm (Kessler 1975).

Immunofluorescence staining. Cells were fixed for 30 min at room temperature in a solution containing 4% freshly prepared paraformaldehyde in PBS at pH 7.2, washed in same buffer, and allowed to adhere for 10 min to coverslips previously coated with 0.1% poly-L-lysine. Subsequently, they were incubated for 30 min in the presence of 50 mM NH_4Cl to block free aldehyde groups, washed in PBS, and incubated for 60 min in the presence of the polyclonal antibody recognizing 70/74 kDa glycoprotein (1:100 dilution in PBS) of endosymbiont-bearing *C. deanei*. Afterward they were washed in PBS-3% bovine serum albumin and incubated in the presence of fluorescein-labeled goat anti-rabbit IgG (EY Laboratories, San Mateo, California, USA) for 60 min (diluted 1:100 in PBS). Next, the specimens were mounted with N-propyl gallate and observed in a Zeiss microscope equipped for fluorescence. Control preparations were incubated without the primary antibody.

Analytical methods. Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as a standard. Neutral sugars were determined with the phenol-sulfuric method, with glucose as a standard (Dubois *et al.* 1956).

RESULTS

Symbiont-containing and symbiont-free strains of *C. deanei* showed very similar SDS-PAGE protein profiles (Fig. 1A). Both strains displayed a large number of bands ranging from 6 to 96 kDa stained with Coomassie Blue. Most of the intensely stained bands were present in the hydrophilic phase. A double band with molecular mass of 40/44 kDa, one major band from 50 to 63 kDa, and one band from 90 to 95 kDa were more evident in endosymbiont-free cells (Fig. 1A, lane 3). Poorly stained proteins were seen in the hydrophobic phases of sym-

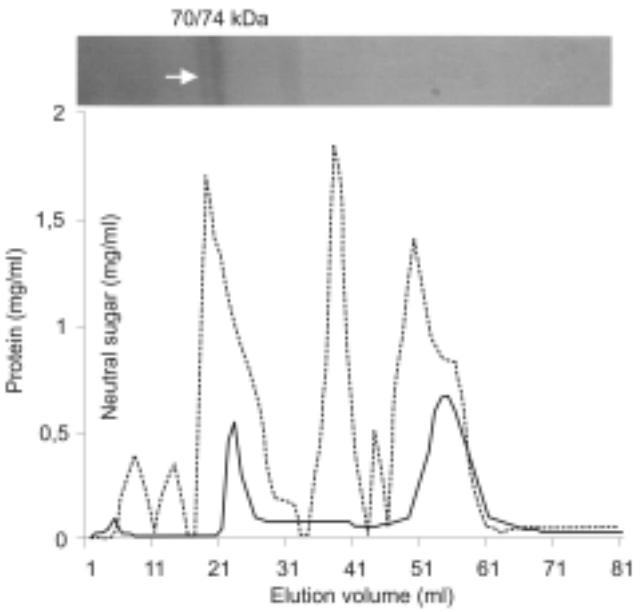
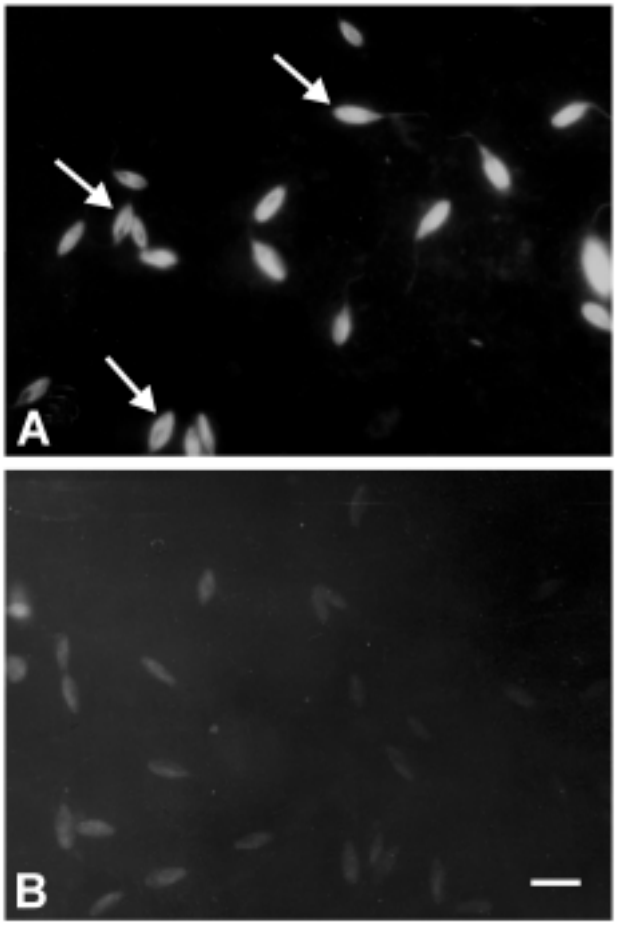


Fig. 2. Gel filtration on a 2 × 120 cm column of Sephadex G-100 of aqueous extracts of symbiont-containing *Crithidia deanei*. Peak I was eluted between 17 to 23 ml at a position of molecular mass from 70-80 kDa, and when examined by polyacrylamide gel electrophoresis (top) showed a double band with a molecular mass of 70/74 kDa, *arrow* major band as described in Results. Fraction size = 5.0 ml.

biont-containing and symbiont-free strains (Fig. 1A, lanes 2 and 4).

A double band with molecular mass of 70/74 kDa was present in the hydrophilic and hydrophobic phases obtained from symbiont-containing *C. deanei* (Fig. 1B, lanes 5 and 6), but absent from symbiont-free cells (Fig. 1B, lanes 7 and 8). Both symbiont-containing and symbiont-free strains exhibited two distinct glycoproteins with masses of approximately 97 and 20 kDa in the hydrophilic phase (Fig. 1B, lanes 5 and 7). Moreover, both strains showed a broad and intensely stained conjugate (less than 20 kDa) near the bottom of the gel in the hydrophobic phase (Fig. 1B, lanes 6 and 8).

Treatment of *C. deanei* with Triton X-114 non-ionic detergent at 0°C, followed by low-speed centrifugation, generated a detergent-insoluble pellet and a detergent-soluble supernatant. The supernatant was further fractionated by phase separation at 37°C into a detergent-rich phase and a detergent-depleted or aqueous phase. The results showed that all the strains of *Crithidia* contained proteins with molecular masses ranging between 6 and 96 kDa. Between the hydrophilic (aqueous) and hydrophobic (detergent-rich) phases, only quantitative differences were observed.



Figs 3A, B. Immunofluorescence microscopy. *Crithidia deanei* were incubated first in the presence of antibodies recognizing 70/74-kDa glycoprotein and subsequently in the presence of FITC-labeled goat anti-rabbit IgG and observed fluorescence microscopy; **A** - intense labeling of the surface of wild strain of *C. deanei* (arrows); **B** - surface labeling was not observed when symbiont-free cells were used. Scale bar 10 µm.

Marked differences in the plasma-membrane glycoproteins between the cured and the symbiont-containing strains of *C. deanei* were observed in the present study, mainly the presence of the double band (70/74 kDa) in both phases obtained from symbiont-harboring *C. deanei*, versus its absence from symbiont-free cells. On the other hand, a glycoconjugate with a low molecular mass (< 20 kDa) was detected in the hydrophobic phases of both symbiont-containing and symbiont-free cells, as shown in Fig. 1B (lanes 6 and 8).

In order to confirm this finding, glycoconjugates were extracted from endosymbiont-bearing *C. deanei* as described in the Material and Methods. The supernatant was lyophilized and the residue fractionated by column

chromatography on Sephadex G-100 (Fig. 2), resulting in the separation of two major peaks monitored for carbohydrate and protein. Peak I was eluted between 17 to 23 ml at a position of molecular mass from 70 to 80, and when examined by polyacrylamide-gel electrophoresis showed a double band with a molecular mass of 70/74 kDa (Fig. 2, top).

Cell localization of 70/74 kDa glycoprotein was performed by using polyclonal antibodies which were detected with FITC-labeled secondary antibodies. Intense labeling of the cell surface of endosymbiont-bearing *C. deanei* was observed in previously fixed cells (Fig. 3A). Cell surface labeling was not observed in symbiont-free cells (Fig. 3B). No labeling was observed when purified glycoprotein was added to the incubation medium, or when cells were incubated only in the presence of FITC-labeled secondary antibodies (data not shown).

DISCUSSION

Evaluation of the total protein profile of trypanosomatid strains by SDS-PAGE is not a particularly useful method, because the strains showed a large number of protein bands of different apparent molecular masses, and they also were very similar among different strains (data not shown). Protein extraction performed with Triton X-114 is a more efficient method to analyze proteins and glycoconjugates in SDS-PAGE, because it requires small amounts of sample and provides clearer profiles, allowing individualization of bands. *C. deanei* displayed a large number of proteins ranging from 6 to 96 kDa. Proteins with molecular masses of 40 kDa, 50/63 kDa, and 90/95 kDa are more evident in endosymbiont-free cells than in endosymbiont-containing cells. d'Avila-Levy *et al*. (2001) demonstrated the absence of the cell-associated cysteine proteinase of 100 kDa and a two-fold enhancement of extracellular proteinases in the cured strain, suggesting that the prokaryote endosymbiont induces alteration in the proteolytic profile in *C. deanei*. Proteinases are enzymes that have been implicated in a number of aspects of host-parasite interactions, including tissue and cell invasion, parasite differentiation, inactivation of deleterious host proteins, and catabolism of exogenous proteins for nutrition purposes (McKerrow *et al*. 1993, Faria e Silva *et al*. 1994, Engel *et al*. 1998, Sajid and McKerrow 2002).

In order to identify and compare the major glycoproteins, the two phases (hydrophobic and hydrophilic) obtained from Triton X-114 extracts of endosymbiont-harboring *C. deanei* and cured with high doses of chloramphenicol were analyzed by SDS-PAGE after staining with Schiff's reagent. Only the wild strain of *C. deanei* contained a glycoprotein with molecular mass of 70/74 kDa. On the other hand, two different glycoproteins with masses of approximately 97 and 20 kDa, were shown by both symbiont-containing and symbiont-free strains, in the hydrophilic phase. Moreover, both strains showed a broad and intensely stained conjugate (less than 20 kDa) near the bottom of the gel in the hydrophobic phase. These results concord with those of previous studies, in which, after analysis of the glycoconjugates of trypanosomatid genera such as *Herpetomonas, Endotrypanum, Leishmania, Trypanosoma* (Branquinha *et al.* 1995), and *Phytomonas* (Abreu Filho *et al.* 2001), glycoconjugates with molecular masses below 20 kDa were observed.

The immunofluorescence assay demonstrated that the glycoproteins of 70/74 kDa, present only in the endosymbiont-harboring *C. deanei*, are located in the plasma membrane. It has been reported that there is a difference in the composition of carbohydrates exposed on the cell surface of endosymbiont-containing and endosymbiont-free strains of *C. deanei* and *Herpetomonas roitmani*, suggesting that the presence of the symbiont bacterium can induce alterations in the surface of the cells (Dwyer and Chang 1976, Esteves *et al.* 1982, Faria e Silva *et al*. 1994). It is interesting that these studies suggest that endosymbiont-bearing *Crithidia* species present fewer surface-exposed carbohydrates than do other species such as *C. fasciculata* and *C. lucillae*, which naturally lack the symbiont. Recently, Fampa *et al*. (2003) showed that an endosymbiont-free strain showed a significant decrease in the interaction with cells and gut tissue from several insect species, compared with the endosymbiont-harboring strain. The reported differences in the cell-surface carbohydrates between endosymbiont-bearing and endosymbiont-free strains imply that these carbohydrates may be involved in the interaction between monoxenous trypanosomatids and cell lines or insect guts. It has been reported that carbohydrates present on the cell surface of *Leishmania* are responsible for the adhesion of the protozoan to the midguts of sandflies (Sacks and Kamhawi 2001). In addition, mouse peritoneal macrophages engulf more endosymbiont-containing than endosymbiont-free protozoans, and the presence of the endosymbiont confers resistance to the macrophage killing mechanisms (Rozental *et al.* 1987).

The major result emerging from the present study was the differences found in the plasma-membrane glycoproteins between the cured and the symbiont-containing strains of *C. deanei*. The presence of the double band (70/74 kDa) in both phases obtained from symbiont-containing *C. deanei*, and its absence from symbiont-free cells, suggests that the symbiont somehow influences the glycoconjugate composition of the plasma membrane in these cells. Further studies using the purified 70/74-kDa glycoprotein may clarify the basic aspects of the role played by the glycoconjugate in the process of the interactions of *C. deanei* with its intracellular symbionts.

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