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Ultrastructural analysis and identification of membrane proteins in the free-living amoeba *Difflugia corona*

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ABSTRACT: Syntaxin-1 and 25-kDa Synaptosome-associated Protein (SNAP-25) are present in the plasma membrane of several different secretory cell types and are involved in the exocytosis process. In this work, the free-living amoeba *Difflugia corona* was studied in relation to ultrastructure, structural membrane proteins, and proteins such as Syntaxin-1 and SNAP-25. Our results obtained by scanning electron microscopy in the amoeba without its theca, showed many membrane projections and several pore-like structures. Using immunocytochemistry, we found structural proteins Syntaxin-1 and SNAP-25.

Introduction

Amoebae are eukaryotic unicellular organisms, belonging to phylum Sarcodina (Lee *et al*., 2000). In most cases, free-living amoebae possess a rigid cover called the theca, which provides protection to the ectoplasm. It is made up of particles captured from the environment, such as grains of sand and diatom frustules, which is the case for the amoeba *Difflugia globosa* (Martínez-Hernández and Silva-Briano, 2003), or it is made up of materials produced by the amoeba itself, like kitin, which is the case for the amoeba *Arcella* (Martínez Pérez *et al*, 2003).

In particular, *D. corona* has a round, oval-shaped, lobulated or dentated oral opening in its theca (Kudo, 1980; Patterson, 1998; Lee *et al.,* 2000; Thorp and Covich, 2001), with in some cases an internal diaphragm and several (two, four or seven) lobes on its rear end. The approximate measurements of *D. corona* individuals in micrometers (including theca) are: 265.6 in width; 234.3 in length without the lobe; and 342.8 in length include the lobe (Fig. 1). However, we have not revised the description of the ultrastructural features of *D. corona* without the theca.

Exocytosis is a process that involves several proteins located in both the plasma membrane and vesicle membrane. Syntaxin-1 and 25-kDa Synaptosome-associated Protein (SNAP-25) are present in the plasma membrane of several different secretory cell types and take part in the binding and fusion of secretory vesicles with the plasma membrane (An and Almers, 2004). Other than in vertebrates, these proteins have been described in invertebrates such as the fruit fly *Drosophila melanogaster* (Wu *et al*., 1999), the cockroach *Leucophaea maderae* (Johard *et al*., 1999), the free-living nematode *Caenorhabditis elegans* (Van-Swinderen *et al*., 1999), and the parasitic worms *Fasciola hepatica, Monieza expansa* and *Ascaris suum* (Quintanar and López, 2001). In relation to the free-living amoeba *D.*

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corona, there is no information on these proteins nor on membrane proteins with antigenic and structural features, as has been described in other amoebae such as *Entamoeba histolytica* (Ventura-Juárez *et al*., 2002). In view of these facts, we studied the ultrastructure of *D. corona* without the theca, using scanning electron microscopy, and the expression of Syntaxin-1, SNAP-25, and structural membrane proteins using immunocytochemical methods.

Material and Methods

Samples

D. corona samples were collected from littoral waters in "El Tecuancillo", located in the Mexican State of Aguascalientes. Samples were fixed in 4% neutral formalin; subsequently, the amoebae were identified and separated for use in different techniques. Several amoebae were maintained with their thecas intacts, so that they could be identified using scanning electron microscopy. Others were placed in acrylic boxes where their thecas were removed with tungsten wire needles, to allow us to perform ultrastructural analysis also using electron scanning microscopy. Another group of amoebae without theca were embedded in paraffin to identify proteins by immunocytochemistry.

Scanning Electron microscopy

Scanning electron microscopy was used to study amoeba with the theca intact. Samples were dehydrated with alcohol (60 to 100%) Extent moisture was removed by liquid CO_2 in a critical point dryer (TOUSIMIS). Samples were then coated with gold using a DESK II chamber, and they were photographed with a JEOL LV 5900 SEM.

The same procedure was used for structural observation of amoebae with the theca removed.

Immunocytochemistry

Amoebae without theca were embedded in paraffin and cut into 5 to10 µm-thick sections. The avidin-biotin-peroxidase method was used for immunodetection of Syntaxin-1, SNAP-25, and structural membrane proteins (Vectastin ABC kit, Dimension Laboratories Inc. CA). The following primary antibodies were used: anti-Syntaxin-1 (anti-Syntaxin-1 polyclonal antibody,

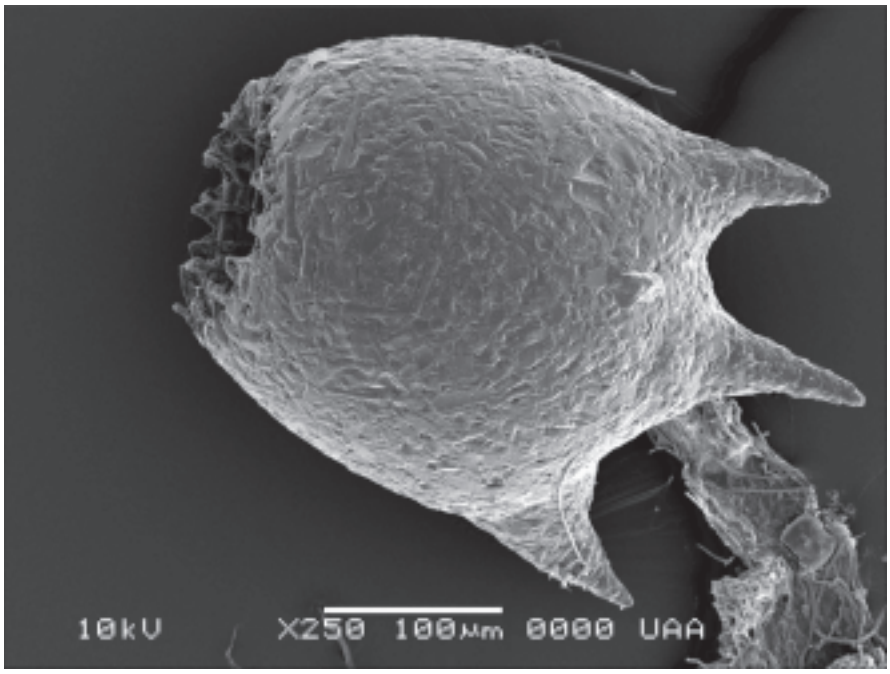

FIGURE 1. Scanning electron microscopy of D*ifflugia corona* with theca. Three of the four lobes are apparent, along with embedded particles of sand and diatoms. The round oral opening can also be seen.

Sigma, St. Louis, MO, USA), diluted 1:200; anti-SNAP-25 (anti-SNAP-25 monoclonal antibody, SMI-81 Sternberg Monoclonal, Baltimore, MD, USA), diluted 1:100; and anti-*Entamoeba histolytica* membrane extracts (polyclonal, developed in rabbit), diluted 1:500 (Ventura-Juárez *et al*., 2002). The samples were incubated with primary antibodies at 4ºC tested for 12 hours. Diaminobenzidine was used to visualize the reaction product. Specificity of immunoreaction was tested by omitting the primary antibody.

Results

Morphological and ultrastructural results obtained by SEM demonstrate that the examined specimens belong to the *D. corona* amoeba species (Fig. 1). Also, *D. corona* without the theca showed peculiar internal morphological features, which include and approximate width of 105.8 microns and a length of 141.1 microns as well as numerous filiform membrane projections (Fig. 2A), and a protoplast with no defined shape. A series of

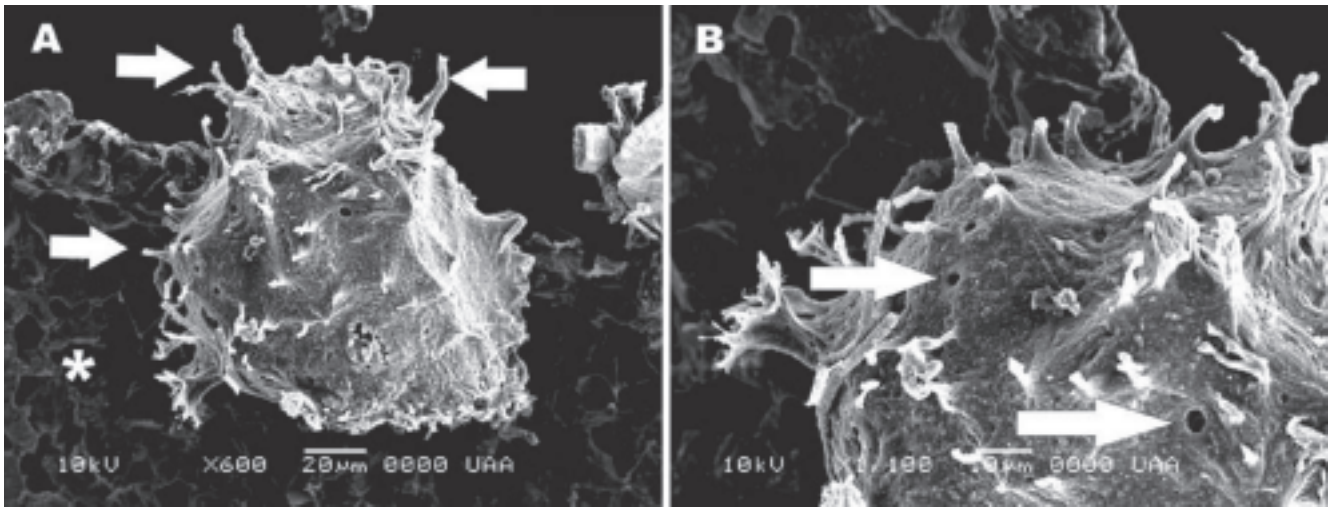

FIGURE 2. Scanning electron microscopy of D*ifflugia corona* protoplast without theca. In **A**, arrows show the numerous membrane projections, and the asterisk indicates a remnant of the theca. In **B**, arrows demonstrate pore-like membrane structures.

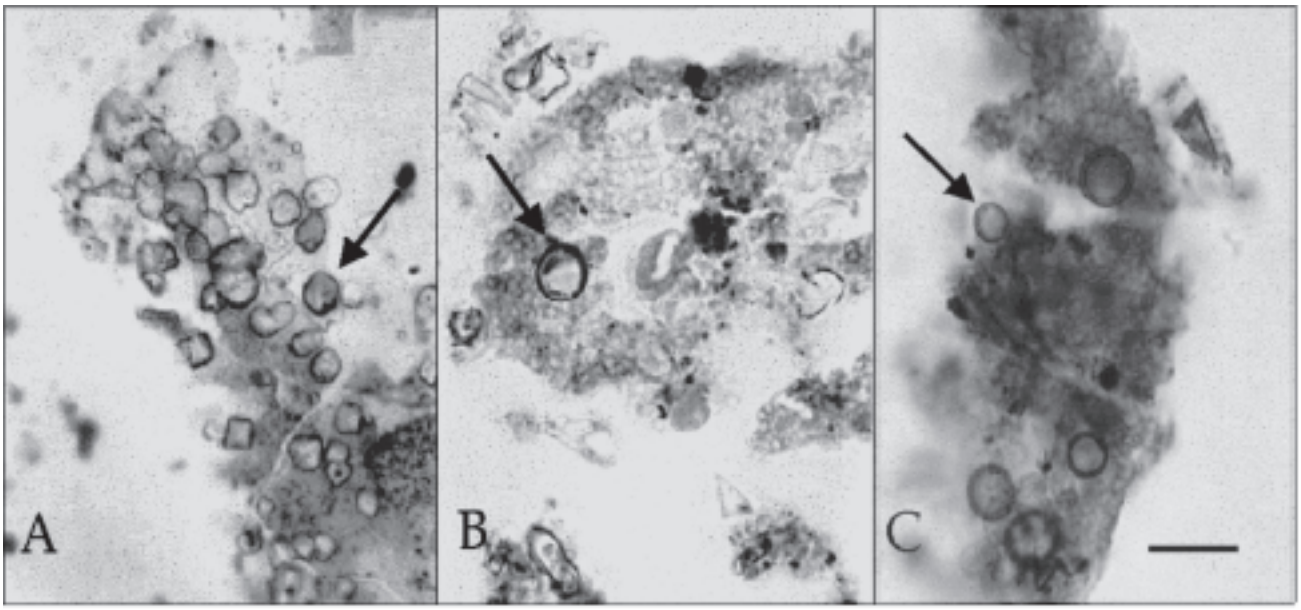

FIGURE 3. Immunopositivity in *Difflugia corona.* Structural proteins (A), Syntaxin-1 (B), and SNAP-25 (C); arrows indicate vacuoles with their immunoreactive membrane (Bar stands for 5 μm).

pore-like structures, up to 45 microns in diameter, were observed on the membrane surface (Fig. 2B).

Using immunostaining for Syntaxin-1, SNAP-25, and structural membrane proteins, we located the immunoreactive material in all samples, in both the plasma membrane and the exocytotic vesicles (Fig. 3).

Immunoreactivity was strong for the three proteins, the strongest for structural proteins. Control slides of amoebae without primary antibody showed no immunoreaction, which confirmed antibody specificity.

Discussion

Our results showed that the free-living amoeba used in this study corresponds morphologically to *D. corona* according to other author's descriptions (Kudo, 1980; Patterson, 1998; Lee *et al.,* 2000; Thorp and Covich, 2001). This species possesses a four-lobed theca, which, judging by its appeareance made up of quartz particles and diatom frustules. In relation to its internal structure, it has irregular protoplast that are similar to those observed in other amoebae such as *Entamoeba histolytica* (Ventura-Juárez *et al*., 2002). The numerous filiform membrane projections observed could represent structures that anchor the plasma membrane to the theca. Also, the *D. corona* possesses pore-like structures in the ectoplasm. Similar structures have been described in the wall of the cyst stage of amoeboflagellate *Naegleria* (Visvesvara *et al*., 2005). However, the pores of amoeboflagellate *Naegleria* were found in the wall, and the pores of *D. corona* are in the plasma membrane. The location of the pores in the plasma membrane may be important in the exchange of substances between the amoeba and the environment. Little information exist on the presence of membrane pores such as those described in the nuclear envelope of other animal cells (Karp, 1996). These observed structures may correspond to temporary holes created by membrane invagination as part of the endocytotic process or membrane renewal.

The expression of Syntaxin-1 and SNAP-25 in the immunocytochemical detection suggests that this proteins take part in the binding and fusion of vacuoles with the plasma membrane to extrude from the cells. It has been demonstrated that secretory vesicles in cells of higher animal species require SNAP-25 for hormone secretion (Kolk *et al*., 2000). On the other hand, immunocytochemical identification of membrane structural proteins in *D. corona* using antibodies against *Entamoeba histolytica* membrane proteins, implied that proteins of this kind are conserved with no difference between a free-living amoeba and a pathogenic one.

In conclusion, we found that the free-living amoeba *D. corona* possesses a membrane with numerous filiform projections and pore-like structures. In addition, using immunocytochemistry, we also determined the presence of exocytosis proteins Syntaxin-1 and SNAP-25 in both the plasma membrane and the vacuole membrane. Lastly, we detected the expression of structural proteins in parasitic amoebae.

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