

Short Communication**Identification of exocytotic membrane proteins, syntaxin-1 and SNAP-25, in *Entamoeba histolytica* from hamster liver**

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Entamoeba histolytica is a protozoan parasite causing dysentery and in some cases liver abscesses. These effects have been attributed to cytolytic substances released by exocytosis. In this study, the presence of the proteins syntaxin-1 and SNAP-25, which are assumed to be involved in exocytosis, were examined by immunohistochemistry, immunoelectron microscopy and western blot analysis. Syntaxin-1 and SNAP-

25 were expressed in the vesicular, vacuolar and plasma membranes of *E. histolytica* trophozoites. It can be concluded that these proteins might be involved in exocytosis processes.

Key words: abscesses, amoeba, exocytosis, parasite, vesicles

INTRODUCTION

ENTAMOEBA HISTOLYTICA IS a protozoan parasite that invades the colonic mucosa producing dysentery and in some cases liver abscesses in humans.¹ The pathogenicity of this parasite is due to cytolytic effects, which have been attributed to exocytotic products such as cystein proteases,² pore-forming proteins,³ collagenases and oligosaccharidases,⁴ as well as neurotransmitter-like compounds.⁵

In higher eukaryotic animals, exocytosis is related to several substances such as neurotransmitters, hormones and excretion products, and the process involves multiple and highly conserved proteins located in the plasmalemma and vesicular membranes of secretory cells. Major progress has been made in identifying these protein components that are essential for docking and fusion of secretory vesicles. These proteins include syntaxin-1 and synaptosome-associated-protein of 25 kDa (SNAP-25) in the plasma membrane, which are considered to be target-specific SNAP receptors (t-SNAREs)

because they interact with the corresponding vesicle-specific SNAP receptors (v-SNAREs) in the vesicle membrane during the formation of the sequential protein complex that triggers secretion/excretion, as has been established in the SNARE hypothesis.⁶

These two proteins were initially considered to be neuron specific.^{7,8} However, it has recently been reported that syntaxin-1 and SNAP-25 are present in invertebrate parasites. Only a few studies have been reported regarding exocytosis and the proteins involved in the process. In fact, the presence of these proteins were observed only in liver fluke *Fasciola hepatica*, ruminant tapeworm *Moniezia expansa* and the nematode *Ascaris suum*.⁹ The present study focused on exocytosis proteins in *E. histolytica* from hamster liver, specifically syntaxin-1 and SNAP-25, which we investigated using immunohistochemistry, immunoelectron microscopy and western blot analysis.

METHODS***E. histolytica* culture, liver inoculation and liver samples**

TROPHOZOITES OF *E. histolytica* HM1:IMSS (México) axenically cultured in TYS-1 medium were used. They were cloned and passed three times through the livers of 10–12 weeks old male golden hamsters (*Mesocricetus auratus*).¹⁰

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In order to carry out liver inoculation, hamsters were injected through the portal vein with 2.5×10^5 trophozoites.¹¹ Animals were killed by anesthesia (sodium pentobarbital, 50 mg/kg i.p.) at 3–6 h postinoculation. Subsequently their abdomen was opened surgically and the portal vein was perfused with a 4% paraformaldehyde, 1% glutaraldehyde, 15% picric acid in 0.1 M sodium cacodylate buffer at pH 7.2, for 30 min. The livers were removed and fixed for an additional 2 h. The samples were embedded in LR-White resin according to the method of Merihgi and Polak¹² for immunoelectron microscopy. For light microscopy, tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections 5- μ m thick were stained with hematoxylin and eosin to identify the presence of amoebas.

Immunohistochemistry of syntaxin-1 and SNAP-25

Paraffin sections 7- μ m thick were used. For the immunohistochemical demonstration of syntaxin-1 and SNAP-25, the avidin-biotin-peroxidase complex method was applied using a Vectastin ABC kit (Dimension Laboratories, CA, USA). Primary antibodies raised against syntaxin-1 (rabbit polyclonal anti-syntaxin-1, Sigma, St Louis, MO, USA) and SNAP-25 (mouse monoclonal anti-SNAP-25, SMI-81, Sternberg Monoclonals, Baltimore, MD, USA) prepared in phosphate-buffered saline (PBS) with bovine serum albumin (BSA) 3% with Triton X-100 (0.025%) were used both at 1:300 dilutions and incubated at 4°C overnight in humidified chamber. The peroxidase reaction was developed with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. The nuclei were lightly counterstained with hematoxylin. The specificity of the immunoreaction was tested by omitting the primary antibody and replacing it with an equivalent concentration of non-specific rabbit immunoglobulin G (IgG). The sections were examined by light microscopy at a magnification of $\times 400$.

Immunodetection of syntaxin-1 and SNAP-25 by electron microscopy

Liver sections 0.5- μ m thick were stained with alkaline toluidine blue for localization of amoeba by light microscopy. Ultrathin sections (80 nm) were obtained using a diamond knife and mounted on nickel grids (Pelco 300, Mesh). Samples were incubated for 2 h with antibodies against syntaxin-1 (diluted 1:50) or SNAP-25 (diluted 1:20). The grids were incubated with goat antirabbit or goat antimouse IgG gold (20 nm)-labeled antibody (Ted Pella, CA, USA) diluted 1:20. Samples

were washed and then incubated with 2.5% glutaraldehyde and stained with uranyl acetate and lead citrate. The samples were examined in a EM-10 transmission electron microscope (Carl Zeiss, Thornwood, NY, USA).

Western blot analyses of syntaxin-1 and SNAP-25

Trophozoites of *E. histolytica* (5×10^7) were washed and suspended in a cocktail of proteases inhibitors (5 mM *N*-ethylmaleimide, 5 mM *p*-chloromercuribenzoate and 1 mM phenylmethylsulfonyl fluoride). Amoebic lysates were obtained by five cycles of freeze–thawing in liquid nitrogen and in water at 37°C. The lysate was centrifuged and the supernatant was collected. Samples enriched in membrane proteins obtained from hypophysis rat were used as positive control specificity to both syntaxin-1 and SNAP-25 and as negative control thymus. Sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (13.5% SDS-PAGE) was carried out using the mini-protean system (Bio-Rad, Hercules, CA, USA). After electrophoresis, gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes (Sigma). The membranes were incubated at 4°C overnight with anti-syntaxin-1 or anti-SNAP-25 antibodies both diluted 1:1000. The membranes were incubated for 2 h with alkaline phosphatase-conjugated secondary antibody diluted 1:20 000 and the alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma).

RESULTS

Immunohistochemistry of syntaxin-1 and SNAP-25

IN ALL LIVER samples, trophozoites were conclusively immunoreactive for syntaxin-1 and SNAP-25. The positivity was concentrated mainly at the cell periphery and around the cytoplasmic vacuoles and vesicles (Fig. 1). All trophozoites observed in the liver were immunoreactive for both proteins. The intensity of immunostaining for SNAP-25 was weak as compared to syntaxin-1. No immunostaining was observed in the control sections without the primary antibody or those replaced with non-specific IgG.

Immunodetection of syntaxin-1 and SNAP-25 by immunoelectron microscopy

The plasma membranes of *E. histolytica* trophozoites were clearly immunogold labeled with syntaxin-1 and SNAP-25 antibodies (Fig. 2). The SNAP-25 immunola-

Figure 1 Microphotographs of liver inoculated with *Entamoeba histolytica* trophozoites. (a) Control without the primary antibody; (b) immunostaining for anti-syntaxin-1; and (c) immunostaining for anti-SNAP-25. Black arrow, trophozoites with immunoreactive material in the plasma, vesicular and vacuolar membranes; white arrow, polymorphonuclear leukocytes; H, hepatocytes. The sections were counterstained with hematoxylin. Bar = 10 µm.

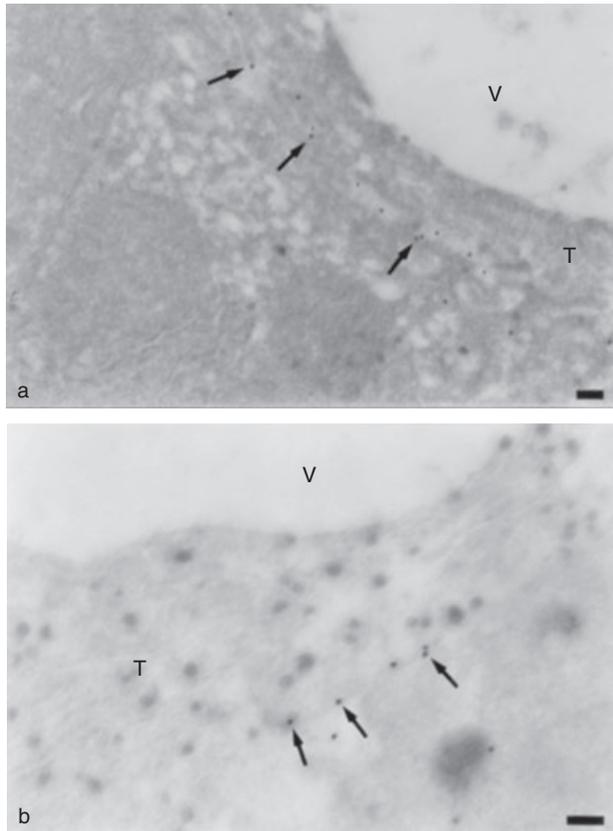
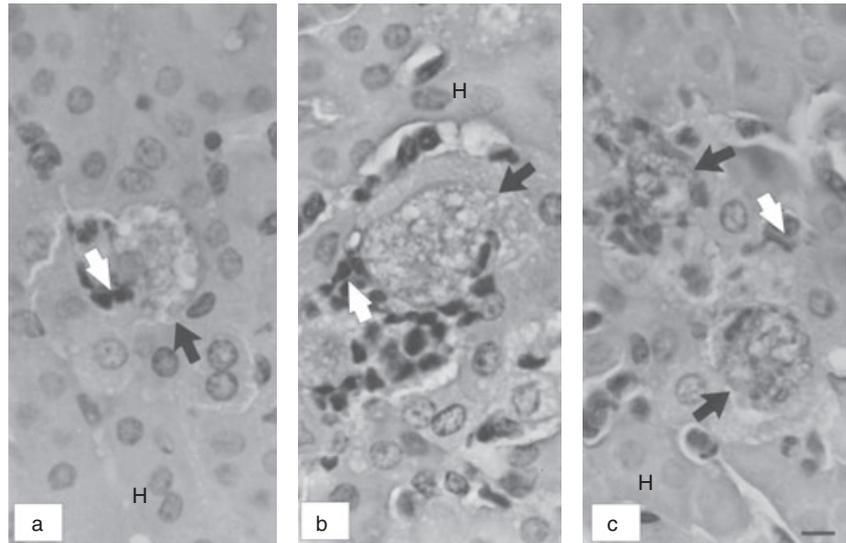


Figure 2 Immunoelectron microscopy of trophozoite in the hamster liver: (a) syntaxin-1, showing multiple gold-labeled particles (arrows) on the plasma membrane; and (b) SNAP-25, showing several immunolabeled particles (arrows) on the plasma membrane. T, trophozoite; V, vacuole. Bar = 0.2 µm.

bel was weak as compared to that of syntaxin-1. No positive label was seen in the membranes of vesicles and vacuoles.

Western blot analyses of syntaxin-1 and SNAP-25

The syntaxin-1 and SNAP-25 antibodies detected two bands at 35 and 25 kDa, respectively, in the immunoblot of trophozoite lysates (Fig. 3). Similar bands were obtained in positive control samples of hypophysis and no bands were observed in thymus (negative control) (Fig. 3).

DISCUSSION

VESICULAR OR VACUOLAR traffic is essential for diverse cellular processes that require membrane

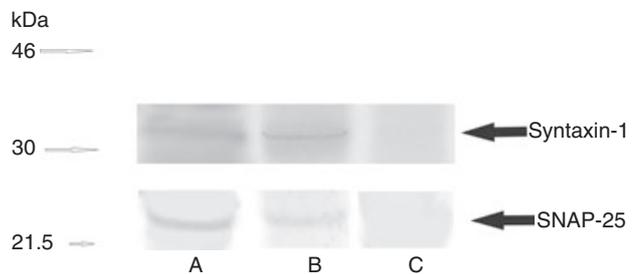


Figure 3 Western blot analysis of syntaxin-1 and SNAP-25. 100 µg of protein/band were processed for SDS-PAGE (13.5%) and electrotransferred to PVDF membrane. A, Hypophysis as positive control; B, lysate from *Entamoeba histolytica* trophozoites; C, thymus as negative control.

fusion during exocytosis of various products. This process is regulated by a protein machinery conserved from yeast to human.¹³ We found that *E. histolytica* trophozoites contained the exocytotic proteins syntaxin-1 and SNAP-25 as shown by immunohistochemistry, immunoelectron microscopy and western blot analysis. The homolog proteins of syntaxin, Sso1 and Sso2, and of SNAP-25, Sec9,¹⁴ have been described in other non-parasitic unicellular organisms, and the yeast *Saccharomyces cerevisiae*, where they participate in a constitutive fashion in the exocytosis of enzymes. The presence of these proteins in the plasma membrane of *E. histolytica* trophozoites might suggest that they are involved in exocytosis of several secretion/excretion products. The biological significance is that some of these products, such as cystein proteinases, amebopore and collagenases, might be capable of lysing cells playing a role in killing intestinal epithelial cells, hepatocytes and host defense cells.

Our study demonstrated by immunohistochemistry syntaxin-1 and SNAP-25 expression in vesicular and vacuolar membranes of trophozoites. Similar results have been reported regarding SNAP-25 in secretory vesicles of pituitary cells,¹⁵ PC12 cells¹⁶ and neutrophil leukocytes.¹⁷ The mechanisms of docking and fusion, in which both proteins are present in the plasma as well as vesicular and vacuolar membranes, remain to be elucidated.

The pathogenicity of *E. histolytica* is due to its cytolytic effects, attributed to exocytotic products. Our results suggest that the process of exocytosis might involve syntaxin-1 and SNAP-25.

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