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A Netrin-3 Like Protein is Secreted from *Tetrahymena thermophila*

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A Netrin-3 Like Protein is Secreted from Tetrahymena thermophila

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Abstract

Netrin proteins are a family of laminin-related secreted proteins that provide signals for axonal growth and cell migration during vertebrate development. Netrin homologs are expressed throughout the animal kingdom; however, some animals do not express a homolog of any known netrin receptors. We have previously found that the ciliated protozoan, *Tetrahymena thermophila*, responds to netrin-1 peptide by showing avoidance behavior. In addition, *Tetrahymena* secrete a protein that is immunologically similar to netrin-1 as detected by ELISA. Since netrin-3, like netrin-1, is a guidance molecule for axons and overlaps signaling pathways with netrin-1 in vertebrates, we hypothesized that netrin-3 may also act as a chemorepellent in *Tetrahymena.* While behavioral assays did not confirm this hypothesis, growth assays indicate that netrin-3 peptide inhibits cell division in *Tetrahymena.* In addition, ELISA and Western blots indicate that a netrin-3 like protein of approximately 48 kDa is secreted from *Tetrahymena.* Immunolocalization of this protein within the cell shows that it appears widely distributed throughout the cell, and co-localizes with the netrin-1 like protein. Using ER tracker™, we found that some of the netrin-3-like protein co-localizes with the endoplasmic reticulum, as might be expected for a secreted protein. Further experimentation is necessary to determine the mechanism by which netrin-3 peptide inhibits growth in *Tetrahymena.*

Introduction

Netrins are a family of proteins belonging to the laminin-related protein superfamily. Several netrins, including netrin-1, netrin-3, and netrin-4 are secreted proteins. Secreted netrin proteins are involved in many developmental processes, such as cell division, cell migration, neuronal guidance, and angiogenesis. The best characterized netrin in this family, netrin-1, signals through five different receptors using a number of different signaling pathways. Netrin-3 shares some signaling apparatus with netrin-1 in vertebrates, and is localized to the axons of motor, sensory, and sympathetic neurons. Because netrin-1 peptide is a chemorepellent in *Tetrahymena* and because *Tetrahymena* secrete a netrin-1 like protein, we hypothesized that netrin-3 might also be biologically active in this organism.

Methods & Materials

Immunofluorescence

Immunofluorescence was carried out using a modified protocol obtained from cellsignal.com. Briefly, cells were washed twice in PBS, reconstituted in 3.7% formaldehyde in PBS, and allowed to fix for 15 min at room temperature. After fixation, cells were rinsed three times in PBS before being blocked in blocking buffer for 60 minutes. After washing off blocking buffer, cells were incubated overnight at room temperature in primary antibody at a dilution of 1:100. After rinsing three times in PBS, cells were incubated in fluorochrome-containing secondary antibody for 1–2 hours at room temperature in the dark. Cells were then rinsed three times in PBS. Ten microliters of cell suspension was then applied to a slide and mixed with one drop of DAPI. Cell suspension was then covered with a coverslip and observed under a fluorescence microscope at 400X.

Western Blotting

Protein extracts were prepared from 2-day old *Tetrahymena* cultures and run on a 10% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and Western blots were performed using a 1:1000 dilution of goat anti-netrin-3 IgG as the primary antibody and a 1:2000 dilution of rabbit-anti-goat IgG, alkaline phosphatase conjugate, as the secondary antibody. NBT substrate was used to show alkaline phosphatase activity.

Results

HDEGGAPRGCVP

Figure 1. Human Netrin-3 Peptide, amino acids 40-51, carries no net charge. This is our best estimate of the sequence we are using based on the information we were given; the sequence is proprietary. Acidic amino acids are shown in red. Basic amino acids are shown in blue. This peptide is not a chemorepellent in *Tetrahymena thermophila.* In constrast, peptides carrying a high net positive charge often elicit avoidance reactions in this organism.

Results

Figure 2. Western Blot of *Tetrahymena* proteins with a polyclonal antibody against netrin-3 shows a band of approximately 48 kDa is present in secreted protein. This band is significantly smaller than human netrin-3, which has a molecular weight of 62 kDa. *Tetrahymena* protein purified by ion exchange chromatography showed no bands on a Western blot, indicating that the netrin-3 like protein is not a basic protein.

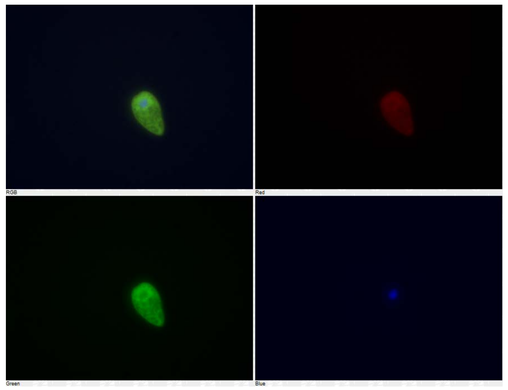

Figure 3. Netrin-3 like protein co-localizes with Netrin-1 like protein in *Tetrahymena*. The top-left photo represents an overlay of all of the stains. Staining with the anti netrin-3 antibody is shown in red. Staining with the anti-netrin-1 antibody is shown in green. Nuclear staining is shown in blue.

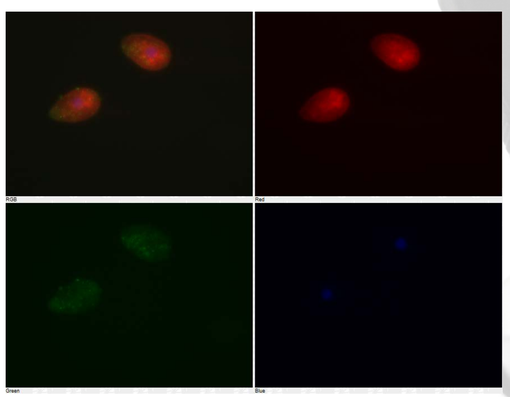

Figure 4. Netrin-3 like protein shows some co-localization with ER Tracker ™ in *Tetrahymena.* The top-left photo represents an overlay of all the stains. Staining of ER Tracker™ is in red, netrin-3 like protein is in green, and nuclear staining is in blue. In addition to intracellular staining, netrin-3 appears to localize on extracellular structures in *Tetrahymena* which are most likely cilia.

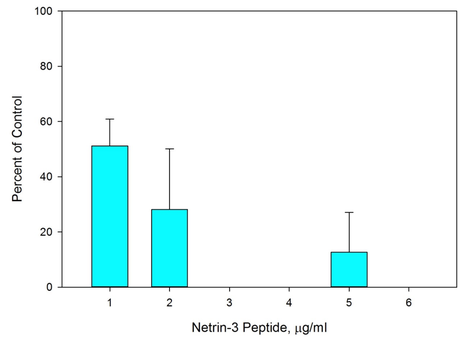

Figure 5. Netrin-3 peptide (C-19) inhibits growth in *Tetrahymena thermophila.* Data are plotted relative to growth of a control culture. All inhibition was highly significant, with a p value of less than 0.0001 in a two-tailed T-test. The N-terminal region of the peptide (H-13) also significantly inhibits growth (data not shown). In contrast to netrin-3 peptide, netrin-1 peptide had no significant effect on growth of *T. thermophila* (data not shown).

Conclusions

- A netrin-3 like protein of approximately 48 kDa is secreted from *Tetrahymena.*
- This netrin-3 like protein co-localizes with the netrin-1 like protein of *Tetrahymena.*
- The netrin-3 like protein colocalizes somewhat with ER Tracker™; however, it also appears to be found on the cilia of the organism.
- Both an N-terminal and a C-terminal netrin-3 peptide inhibits mitosis in *Tetrahymena.* The netrin-3 like protein may be a mitotic regulator in *Tetrahymena thermophila.* Future experiments including viability assays are necessary to determine whether netrin-3 plays a role as an antagonist of mitotic signaling in this organism.

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