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Further Biochemical Evidence for Secretion of a Netrin-1 Like Protein from *Tetrahymena thermophila*

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Further Biochemical Evidence for Secretion of a Netrin-1 Like Protein from *Tetrahymena thermophila*

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Abstract

Netrin-1 is a pleiotropic signaling molecule first characterized in its role as an axonal guidance molecule. Since then, additional physiological roles for netrin-1 have been found, implicating netrin signaling in processes such as angiogenesis and tumor progression. Netrins are expressed throughout the animal kingdom. We have previously found that *Tetrahymena thermophila* show avoidance to netrin-1 peptide, and that secreted proteins from *Tetrahymena* show evidence of netrin-1 activity when assayed by ELISA. In our current study, Western blotting using a polyclonal antibody against netrin-1 showed that a protein of approximately 52 kDa was present in both whole cell extract and secreted protein obtained from *Tetrahymena thermophila*. Ion exchange chromatography using CM-Sepharose allowed us to isolate a protein of the same molecular weight, suggesting that this netrin-1-like protein is a basic protein, similar to its mammalian homologue. Immunolocalization using the same antibody showed co-localization of the netrin-1 with the endoplasmic reticulum when counterstained with ER Tracker™, as would be expected for a secreted protein. Since the *Tetrahymena* genome has been sequenced, we hope to purify enough of this protein to obtain an amino acid sequence and confirm the identity of this protein.

Introduction

Netrin-1 is a pleiotropic signaling protein, with roles in axonal guidance, angiogenesis, and cell migration. This protein is currently being experimented with as a chemotherapy agent for glioblastoma and a treatment for myocardial infarction. Netrin-1 blockers are being used to prevent metastasis in some tumor types.

We have previously shown that netrin-1 peptide is a chemorepellent in *Tetrahymena thermophila.* We now present evidence that *T. thermophila* secrete a netrin-1 like protein of approximately 52 kDa, which is smaller than the human analog (70-84kDa). Like human netrin-1, this protein is basic, and is abundant in secreted protein extracts obtained from this organism. In addition, this protein co-localizes with ER Tracker, as would be expected for a secreted protein. Unlike in humans, however, netrin-1 does not significantly impact mitotic rate in *Tetrahymena* at the concentrations we tested (1, 2, and 5 μM).

Materials and Methods

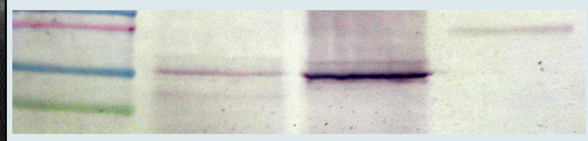
Immunofluorescence

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 1:5000 dilution of goat anti-netrin-1 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.

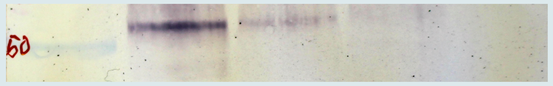
Results



Standards | **Whole Cell Extract** | **Secreted Protein** | **Netrin Control**

Figure 1. Western Blot of *Tetrahymena* proteins with a polyclonal antibody against netrin-1 shows a band of approximately 52 kDa is present in whole cell extract as well as in secreted protein. This band appears to be enriched in secreted protein. This band is significantly smaller than the human netrin control, which runs at 75-80 kDa.

Results



Standards | IEP, pH 7.0 | IEP, pH 8.0 | IEP, pH 9.0 | IEP, pH 10.0

Figure 2. Western blotting of fractions obtained from ion-exchange chromatography with CM-Sepharose indicate that our netrin-1 like protein is a basic protein. The netrin-1 like protein was most abundant in the first fractions eluted from the ion exchange column. This protein also ran at approximately 52 kDa.

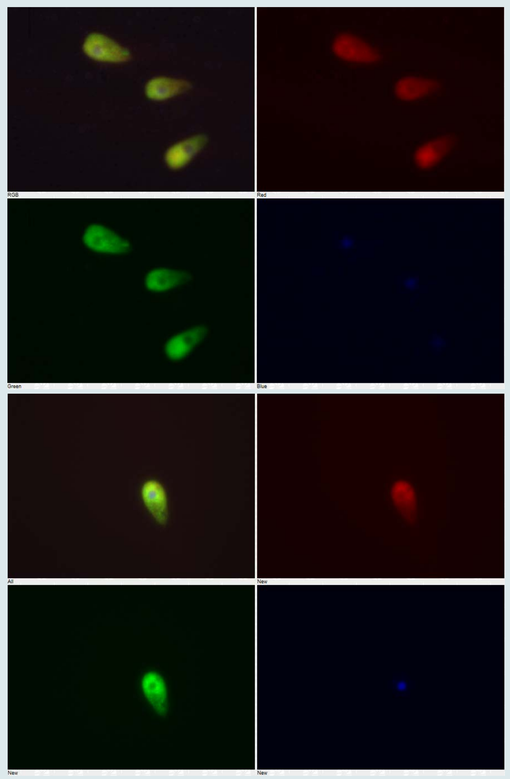


Figure 3. Netrin 1-like protein co-localizes with ER Tracker™, as would be expected for a secreted protein. The top-left photo in each grid represents an overlay of all of the stains. ER Tracker is shown in red. Staining with the anti-netrin-1 antibody is shown in green. Nuclear staining is shown in blue.

Conclusions

- The netrin-1-like protein in *Tetrahymena* is a basic protein with a molecular weight of approximately 52 kDa.
- This netrin-1 like protein is secreted from *Tetrahymena* and is synthesized using the ER trafficking pathway.
- Since netrin-1 is a chemorepellent in *Tetrahymena*, it is possible that cells use their netrin-1 like protein to communicate with one another, allowing cells to spread out and not overgraze an area.

Acknowledgements

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