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Biochemical Evidence for Netrin-Signaling Homologues in Tetrahymena thermophila

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Biochemical Evidence for Netrin-Signaling Homologues in *Tetrahymena thermophila*

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Abstract

Netrins are pleiotropic guidance proteins that are involved in developmental signaling of branched structures within vertebrates. However, like many developmental pathways, dysregulation of the netrin pathway has been implicated in cancer progression and metastasis. Since *Tetrahymena* respond to guidance proteins, showing chemoattractant and chemorepellent behavior, we hypothesized that we could use these organisms as a model system for cancer signaling. We have previously found that netrin-1-peptided, netrin-3-peptide, and recombinant netrin-4 are all chemorepellents in this organism. Since netrin-1-peptide signals through a tyrosine kinase in *Tetrahymena*, we hypothesized that *Tetrahymena* might possess tyrosine kinases as well as a receptor homologous to UNC-5, a netrin receptor which relays signals via tyrosine kinases in vertebrates. Using immunoprecipitation with a polyclonal anti-UNC-5-B antibody, we purified a 250 kD protein from *Tetrahymena* whole cell extract. Similarly, we immunoprecipitated several proteins, including a 60 kD protein and a 75 kD protein using a polyclonal anti-src-antibody. Our purified samples were sent out for identification by mass spectroscopy. Mass spectroscopy indicated that we have purified a number of novel peptides not currently found in the *Tetrahymena* Genome Database. Our data indicate that the proteome database in this organism is incomplete, and that there are additional proteins waiting to be discovered in this organism.

Materials and Methods

- For netrin-like proteins, whole cell extract from *Tetrahymena thermophila* was run on a 10% SDS-PAGE and probed with polyclonal netrin-1, netrin-3, and netrin-4 antibodies. A netrin-like-protein of approximately 50 kD was identified. This band was removed from the gel and sent to Alphalyse for identification by mass spectroscopy.
- For src-like proteins, an immunoprecipitation was run on whole cell extract using an anti-src polyclonal antibody. Immunoprecipitate was then run on a 4-20% SDS-PAGE gradient gel. Several proteins were identified by Western blot with molecular weights between 37 and 75 kD. All of the proteins in this range were removed from the gel and sent to Alphalyse for identification by mass spectroscopy.
- For netrin receptor-like proteins, immunoprecipitations were run on whole cell extract using anti-UNC-5B and anti-neogenin antibodies. Immunoprecipitate was run on a 7.5% SDS-PAGE and a 250 kD band was identified. This protein was removed from the gel and sent to Alphalyse for identification by mass spectroscopy.
- **All amino acid sequences obtained from mass spectroscopy were "BLAST" searched in the Tetrahymena genome database as well as the Uniprot database in order to identify candidate proteins.**

Results

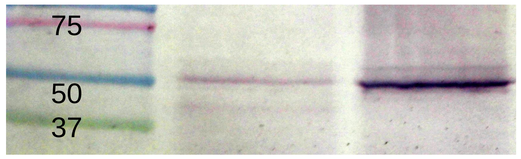

Figure 1. Western blot identifying 50 kD netrin-like protein that was sequenced by mass spectroscopy.

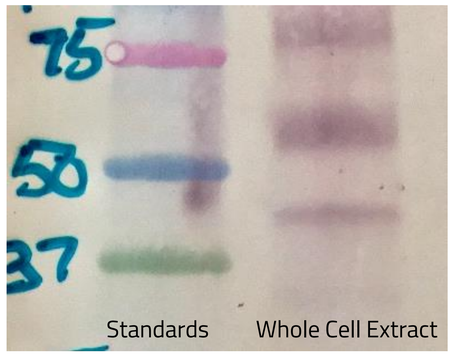

Figure 2. Western blot identifying multiple src-like proteins between 75 and 37 kD that were sequenced by mass spectroscopy.

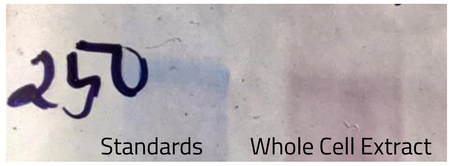

Figure 3. Western blot identifying 50 kD netrin-like protein that was sequenced by mass spectroscopy.

Table 1. Mass spectrometry yielded 56 peptide sequences from putative netrin-like proteins in *Tetrahymena.* These were derived from our 50 kD gel band. Database analysis indicated that most of the proteins had been previously identified in *Tetrahymena*. Only the novel sequences are listed below.

Novel Amino Acid Sequences from 50 kD netrin-like protein sample	
TIDPLSR	ALLEEAVR
ELSDYLK	ITWTIAR
SFLENVVR	YSVSPVVR
VLQA IVIR	QVVVDALK

Sequencing Results

Table 2. Mass spectrometry produced 91 peptide sequences from putative netrin-like proteins in *Tetrahymena.* These were derived from our gel slice containing 37-75 kD proteins. Database analysis indicated that most of the proteins had been previously identified in *Tetrahymena,* although two novel sequences, ELII GDR and TSTTVDLK, were identified. Some sequences were identified as proteins from other species, indicating that *Tetrahymena* may have homologs of these proteins. Proteins in the expected molecular weight range are shown in red.

Proteins Identified	Molecular weight and location
Lactotransferrin (*Homo sapiens*)	80 kD; secreted
Zymogen granule protein 16 homolog B (*Homo sapiens*)	22.7 kD; Secreted Jacalin-type lectin
Zinc-alpha-2-glycoprotein (*Homo sapiens*)	34.2 kD; secreted
POTE ankyrin domain family member I (*Homo sapiens*)	122 kD; secreted and exosomal
Prolactin-inducible protein homolog (homologous to several species)	17 kD; secreted
Lysozyme C (*Pan troglodytes)*	17 kD; secreted
Dermcidin (*Homo sapiens*)	11 kD; secreted
Polymeric immunoglobulin receptor (*Homo sapiens*)	83 kD; class I transmembrane
Lipocalin-1 (*Homo sapiens*)	19 kD; secreted
Putative lipocalin 1-like protein 1 (*Homo sapiens)*	18 kD; secreted

Table 3. Mass spectrometry yielded 25 peptide sequences from putative netrin-receptor-like proteins in *Tetrahymena.* These were derived from our 250 kD gel band. Database analysis indicated that most of the proteins had been previously identified in *Tetrahymena,* although two novel sequences, YLYEIAR and AEFVEVTK, were identified. Some sequences were identified as proteins from other species, indicating that *Tetrahymena* may have homologs of these proteins. Proteins in the expected molecular weight range are shown in red.

Proteins Identified	Molecular weight and location
Dermcidin (*Homo sapiens*)	11 kD; secreted
Hornerin (*Homo sapiens*)	282 kD; cytosolic

Conclusions

- Neither SDS-PAGE nor immunoprecipitation resulted in purification of a single protein. However, sequencing of a single 250 kD protein obtained by immunoprecipitation yielded fewer proteins, and presumably a cleaner preparation, than sequencing of a single 50 kD band obtained from whole cell extract.
- None of our database searches identified our candidate proteins as homologs of netrins, src, UNC-5, or neogenin.
- Some of the peptides could not be identified in the *Tetrahymena* Genome Database but were identified in Uni-Prot (100% similarity).
- Some of the peptides could not be identified in either the *Tetrahymena* Genome Database or Uni-Prot. These unidentified peptides could be fragments of previously unidentified proteins from *Tetrahymena thermophila.*
- Many of the proteins we identified were not in the molecular weight ranges of the bands that we excised from the gel, indicating that homologous proteins, if they exist in *Tetrahymena*, are likely quite different from their mammalian homologs.
- Additional analysis will be done on the putative mammalian homologs that were identified to determine whether any homologs can be found in the *Tetrahymena* Genome Database.

Acknowledgements

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