Role of the *Caenorhabditis elegans* Extracellular Matrix Protein F-Spondin/SPON-1 in Neural Development

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Abstract

Extracellular cues are important in many aspects of development including proper morphology and axon guidance. In a genetic screen for mutants with morphological defects during embryogenesis, *spon-1*, the *Caenorhabditis elegans* sole member of the Spondin family of secreted extracellular matrix proteins was identified. The SPON-1 protein is secreted and localizes to multiple basement membranes where it functions to maintain muscle to extracellular matrix attachments. We show here that SPON-1 is also required for proper axon outgrowth, fasciculation and guidance. *spon-1* mutants also display defects in cell migration. We have characterized genetic interactions of SPON-1 with integrin cell matrix receptors. Preliminary data suggest that SPON-1 has antagonistic functions to the INA-1 alpha integrin.

Introduction

Many processes throughout development involve interactions between tissue layers that are mediated by the extracellular matrix (ECM). The response of a cell to ECM cues depends on its repertoire of cell surface receptors sensitive to them. Morphogenesis in the developing embryo requires cues from the ECM to generate the shape of tissues and ultimately the characteristic form of an organism. Neural cells depend upon cell-ECM interactions for proper cell body migration and the outgrowth and maintenance of axon positions. It is becoming increasingly apparent that both axon guidance and morphogenesis use common molecular pathways in cell-ECM interactions (Hinck et al., 2004). Thus understanding the composition and function of the ECM and its components is essential for analysis of morphogenetic processes and axon guidance.

Spondins are a family of highly conserved secreted ECM proteins that contain two subfamilies, Mindins and F-Spondins, which have similar yet distinct protein domains (Feinstein et al., 2004). F-Spondin is thought to function through cell-ECM interactions. The attachment of F-Spondin to the ECM is mediated by the binding of the thrombospondin type 1 (TSR) domains to proteoglycans in vertebrates (Feinstein et al., 2004) and may also mediate cell-ECM interactions in invertebrates as well (Woo and Chisholm unpublished). Most organisms contain members from both subfamilies which are often expressed and potentially function redundantly. As a result, *in vivo* functional
analysis has been difficult in the past. Based on domain organization and sequence, spon-
1 most resembles F-Spondins, which makes C. elegans an excellent model organism to
analyze the function of F-Spondin in vivo.

Analyses in cell cultures suggest that F Spondins have a role in neural and
ovarian cancer development. F-Spondin was originally isolated from rat floor plate(Klar
et al., 1992), which is a group of cells that provide signals required in spinal cord
development. Evidence suggests it may function in axonal regeneration after nerve injury
in adults (Burstyn-Cohen et al., 1998; Burstyn-Cohen et al., 1999; Feinstein et al.,1999).
In human ovarian cells, over expression of a human F-Spondin homologue, vascular
smooth muscle growth-promoting factor, was observed in ovarian carcinomas but not in
normal ovarian epithelium, linking F-Spondin function to ovarian cancer (Pyle-Chenault
et al.,2005).

Previous work in the Chisholm lab has shown that SPON-1 is required for proper
morphology during C.elegans embryogenesis. Furthermore, it is required continuously
for axon formation and maintaining axon position after initial outgrowth. spon-1 mutants
have body constrictions caused by defects in muscle to ectoderm attachment and display
progressive axon guidance defects along the midline as well as defects in anterior lateral
microtubule (ALM) and hermaphroditic specific(HSN) neurons. The molecules SPON-1
interacts with to influence neuronal development have yet to be identified. Candidate
signaling molecules include extracellular proteins and cell surface receptors.

Possible extracellular signaling candidates are members of a disintegrin and
metalloprotease (ADAM) and Wnt proteins. Wnt proteins are involved in multiple
signaling pathways through concentration dependant cell nonautonomous interactions.
The are required in orienting cell and planar polarity and directing cell migration during
early embryonic development. Wnts have recently been identified as axon guidance cues
in both vertebrates and invertebrates. In vertebrates, Wnt repulsion regulates posterior
directed growth of the spinal tract (Liu et al., 2005) and medial-lateral retinotectal
topographic mapping (Schmitt et al., 2005). Similar to spon-1 mutant phenotypes,
C.elegans with deficiencies in the Wnt protein EGL-20, have displaced ALM and HSN
neurons (Zinovyeva et al.,2005). Misplaced axons and cell migration defects are also
apparent in animals deficient in the ADAM protein UNC-71. ADAM proteins are
important regulators of cellular adhesion and recognition through proteolysis and
modular cell adhesion (Huang et al., 2003). Genetic analysis has allowed us to uncover
the effects members of Wnt and ADAM extracellular proteins have on the F-Spondin
signaling pathway.

SPON-1 is hypothesized to diffuse and bind to multiple cell surface receptors.
Possible target receptors include members from the integrin and LDL cell surface
receptors. INA-1 is an integrin which functions as a cell surface receptor of the
extracellular matrix. Genetic analysis in worms, flies and vertebrates has shown
conserved functional roles in providing traction to migrating cells, and transmitting
guidance signals that direct cells to their targets. Integrins also contribute to most
morphogenetic events including neural development and are essential for tissue integrity
(Bokel et al., 2002; Brown et al., 2000). Other possible SPON-1 receptors are the LDL
cell surface receptors APL-1 and T13C2.6. APL-1 is the single homologue of the human
amyloid precursor protein (APP). APL-1 is expressed in the developing nervous system
and is essential for larval viability (Hornsten,A. unpublished). T13C2.6 is a homologue
of the human apolipoprotein E receptor (ApoEr2). Mutant T13C2.6 animals have morphological phenotypes that resemble those of spon-1 mutants. *In vitro* studies suggest that F-Spondin interacts with ApoER2 which in turn affects processing of the APP protein associated with neurodegenerative Alzheimer’s disease.

Here we present data showing that SPON-1 functions through interactions between components of cell surfaces and the extracellular matrix in neural development. Our results yield the first genetic model for the F-Spondin family and characterize F-Spondin functions *in vivo*.

**Materials and Methods**

**Strains**

All *C. elegans* strains were generated from Bristol N2 and grown at 20 ºC on NGM plates under standard conditions (Brenner 1974) unless otherwise stated. The following mutations were used and received from the Chisholm lab: spon-1(ju430) ts, spon-1(e2623), ina-1(gm144), egl-20(n585) ts, unc-71(ju156), Psra-6-GFP(oyIs14), PtpH-1-GFP(zdIs13), Pmec-4-GFP(zdIs5), spon-1(ju430); Psra-6-GFP(oyIs14), spon-1(e2623); Psra-6-GFP(oyIs14), spon-1(ju430); PtpH-1-GFP(zdIs13), spon-1(e2623); PtpH-1-GFP(zdIs13), spon-1(ju430); Pmec-4-GFP(zdIs5) and spon-1(e2623); Pmec-4-GFP(zdIs5). All mutations are recessive with the exception of the transgenes. Strains containing the spon-1(ju430) ts mutations were grown at 15ºC. We used the GFP-marked balancer chromosome mIn1 mIs14 (Edgley and Riddle, 2001) to balance spon-1 alleles.

**Axon guidance analysis**

All axons were observed using epifluorescence microscopy (Zeiss Axioplan with GFP filter sets) at 40x magnification. Transgenes used to examine axon morphology:

<table>
<thead>
<tr>
<th>Transgene</th>
<th>Neural Expression</th>
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<tbody>
<tr>
<td>Psra-6-GFP(oyIs14)</td>
<td>midline (PVQ)</td>
</tr>
<tr>
<td>PtpH-1-GFP(zdIs13)</td>
<td>hermaphroditic specific neuron (HSN)</td>
</tr>
<tr>
<td>Pmec-4-GFP(zdIs5)</td>
<td>anterior lateral microtubule (ALM)</td>
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Transgenic markers were introduced into ina-1(gm144), egl-20(n585) ts and unc-71(ju156) by mating N2 males containing the GFP transgene with homozygous mutant hermaphrodites. We picked green hermaphrodites in the F1 generation (mutant/++;GFP/+), then picked homozygous transgenic mutants based on phenotype and color in the F2 generation.

**Temperature Shift Assay**

Animals homozygous for the spon-1(ju430) temperature sensitive allele were assayed. Adult hermaphrodites were lysed in a solution of 10% bleach and 10% 10M NaOH and kept at the permissive temperature 15 ºC to synchronize development of the F1 progeny. The F1 progeny were kept at 15 ºC until larval stage 1 and were shifted to
the restrictive temperature 25 °C to develop to adulthood. Axons were scored in young adults.

**Double mutant construction**

Double mutants containing GFP transgenic neural markers were generated using chromosome balancer *mln1 mls14* for all *spon-1* containing strains. The *mln1 mls14* phenotype was used to identify and confirm the presence of the *spon-1* mutation during construction. Homozygous *mln1 mls14* animals have a dumpy (dpy) phenotype and express GFP in the pharynx. Heterozygous animals lack the dpy phenotype and retain pharyngeal GFP expression. N2 males were mated with *mln1 mls14* hermaphrodites. Non-dpy F1 males that expressed GFP in the pharynx (*mln1 mls14/+) were picked and mated with either *ina-1*(gm144) or *egl-20*(n585) mutants containing a GFP neural transgene. F2 males that expressed GFP in both pharyngeal and neuronal cells (*mln1 mls14/+; mutant/+;GFP neural marker+/+) were mated with *spon-1* mutants that contained GFP neural transgenes. Animals that were heterozygous for *mln1 mls14* and homozygous for either *egl-20*(n585) or *ina-1*(gm144) were picked in the F3 generation. Mutants homozygous for either *ina-1*(gm144) or *egl-20*(n585) were identified by picking animals with the protruding vulva (pvl) phenotype characteristic of *ina-1*(gm144) homozygous mutants, or that displayed the homozygous *egl-20*(n585) egg laying defect (egl). We confirmed that *spon-1* was balanced by *mln1 mls14* in the F3 generation by screening the F4 generation for animals with variable abnormal (vab) morphological defects characteristic of *spon-1* homozygous mutants. Double mutants were identified in the F5 generation by picking animals that did not express GFP in the pharynx and were homozygous for GFP neuronal cells.

Double mutants generated include:

<table>
<thead>
<tr>
<th>Strain</th>
<th>Strain number</th>
</tr>
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<tbody>
<tr>
<td><em>spon-1</em>(ju430);<em>ina-1</em>(gm144);Ptph-1-GFP(zdIs13)</td>
<td>CZ6217</td>
</tr>
<tr>
<td><em>spon-1</em>(ju430);<em>ina-1</em>(gm144);Psra-6-GFP(oyIs14)</td>
<td>CZ6290</td>
</tr>
<tr>
<td><em>spon-1</em>(ju430);<em>egl-20</em>(n585); Psra-6-GFP(oyIs14)</td>
<td>CZ6293</td>
</tr>
</tbody>
</table>

**Results**

**SPON-1 and EGL-20 may act in parallel pathways**

SPON-1 and EGL-20 are both components of the ECM that are required for proper axon guidance. In order to determine if an interaction between SPON-1 and EGL-20 is required for neuronal development, we analyzed PVQ midline neural cells in single and double mutants. *spon-1;ina-1* double mutants displayed more penetrant defects than *spon-1* and *egl-20* mutants(fig. 1F). This may be an additive affect and
suggests that \textit{spon-1} and \textit{egl-20} act in parallel pathways and \textit{spon-1} axon guidance is not \textit{egl-20} dependant in the midline.

**SPON-1 is required for integrin dependant cell adhesion in the midline during early stages of development**

Cell adhesion via F-Spondins and integrins are required for proper axon guidance. Both \textit{spon-1} and \textit{ina-1} mutants have defects in midline crossing (fig.1). We examined whether F-Spondins affect integrin dependant cell adhesion in the midline through \textit{spon-1};\textit{ina-1} double mutant analysis. \textit{spon-1} suppresses the \textit{ina-1} midline crossing phenotype suggesting that \textit{spon-1} acts as an \textit{ina-1} inhibitor (fig.1C).

We next examined when \textit{spon-1} affects \textit{ina-1} (fig1C). We performed a temperature shifting assay on a temperature sensitive \textit{spon-1} allele, \textit{ina-1}, and in \textit{spon-1};\textit{ina-1} double mutants. Midline crossing defects were more penetrant in \textit{spon-1} mutants that developed as embryos at 15 °C and raised to 25 °C at larval stage 1, than those kept at 15 °C. These findings suggest that SPON-1 is required throughout development. On the contrast, the percentage of crossing defects in \textit{spon-1};\textit{ina-1} mutants kept at 15 °C was similar to those raised to 25°C, suggesting \textit{spon-1} may affect \textit{ina-1} during early development.
Percentage of animals with midline crossing defects

E

Percentage of animals with midline crossing defects (kept at 15 °C)

F

\[ \text{Percentage} \]

\[ \begin{array}{c}
\text{N2} \\
\text{spo-1(ju430)} \\
\text{par-1(aj44)} \\
\text{spo-1(ju430);par-1(aj44)} \\
\end{array} \]
Fig. 1. Midline Crossing Phenotype of PVQ neurons in *spon-l* and *ina-l* single and double mutants. (A-D) D-V view of *oyIs14[Psra-6-GFP]* in wild type and *spon-l(ju430);ina-l(gm144)*. Panel A shows a 10x magnification from head to tail, whereas (B-C) shows a 40x magnification of the mid-body to tail. In wild type and *egl-20* mutants (A-B) processes overlap at the posterior (red arrowhead) and extend along the midline separately to the anterior. In some *spon-l(ju430);ina-l(gm144)* double mutants (C), along with *spon-l(ju430)* and *ina-l(gm144)* single mutants, midline processes overlap and then separate as they extend to the anterior. In some *ina-l* mutants (D) midline processes overlap and adhere together as they extend to the anterior. We quantified the midline crossing defects observed in *spon-l* and *ina-l* (E) in animals that were kept at 15 °C and shifted to 25 °C after initial outgrowth in larval stage 1. The percentage of *spon-l* mutants kept at 15 °C (n = 51) with midline crossing defects was 13.7%. The percentage of animals with midline defects increased to 39.1% (n = 64) when L1s were shifted to 25°C (p< 0.005). 76.0% of *ina-l* mutants kept at 15 °C (n = 25) and 88.2% kept at 25 °C (n = 17) displayed this phenotype. *spon-l;ina-l* double mutants were 26.1% (n = 23) at 15 °C and 33.3% defective (n = 9) at 25 °C. We quantified midline crossing defects in *spon-l* and *egl-20*. No crossing defects were observed in N2 or *egl-20*. 13.7% of *spon-l* and 22.7% of *spon-l;egl-20* mutants showed midline crossing defects.

The effect of SPON-1 on integrin dependant cell body migration

F-Spondin and integrin mediated cell adhesion is necessary for HSN cell body migration (fig. 2). HSN cell bodies normally migrate from the posterior end to the vulva (fig. 2A) and fail to migrate in *spon-l* and *ina-l* mutants (fig. 2B). We analyzed the affects *spon-l* has on *ina-l* cell body migration in double mutants. *spon-l* suppresses the *ina-l*migration defect, which like our previous results, suggests *spon-l*may inhibit *ina-l*. 
Fig. 2. Cell body migration phenotype in spon-1 and ina-1 single and double mutants. (A-B) Lateral view at 10x magnification of expression patterns of zdIs13[PtpH-1-GFP] as seen in the HSNs. In wild type animals (A) all cell bodies (red arrowhead) migrate from the posterior to the vulva. Some cell bodies under migrate in spon-1, ina-1 and spon-1;ina-1 mutants (B). In N2 worms, all cell bodies migrate to targeted positions (C). 6.7% of the cell bodies in spon-1 (n = 178), 68.2% in ina-1 (n = 44) and 2.1% in spon-1;ina-1 mutants (n = 178) fail to migrate to targeted positions (p<0.001 comparing N2, spon-1 and spon-1;ina-1 to ina-1).

Discussion

In this study, we examined the effect F-Spondin has on neuronal development through interactions between components of the ECM. F-Spondins may interact with other components of the ECM to regulate binding to cell surfaces. We tested the affect of F-Spondin on ECM components of the Wnt signaling pathway. Our results suggest that if SPON-1 is involved in the Wnt signaling pathway, the interaction is required downstream of EGL-20.

We also examined interactions between F-Spondins and cell surfaces. We have demonstrated for the first time in vivo that one of the physiological functions of F-Spondins is to regulate integrin dependant cell migration and adhesion. In support of these findings, in vitro studies in human umbilical vascular endothelial cell lines suggest that VSGP/F-Spondin acts as a functional blockade to inhibit integrin adhesion (Terai et al., 2001). The mechanism that mediates this antagonistic effect is not yet clarified.
F-Spondins plays an important role in neuronal cells from early development to adulthood. However, our results indicate that F-Spondin dependent inhibition of integrins may be required during early developmental stages and not during later stages. This suggests F-Spondins signal in different pathways and may bind to multiple receptors.

Acknowledgments

We thank the members of Chisholm and Jin labs for advice, support and help.

References


adhesion and neurite extension. *Cell* 69, 95-110.


