Regulation of TGF-β superfamily signaling by two separable domains of glypican LON-2 in C. elegans

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Regulated intercellular signaling is critical for the normal development and maintenance of multicellular organisms. Glypicans have been shown to regulate signaling by TGF-β, hedgehogs and Wnts in several cellular contexts. Glypicans comprise a conserved family of heparan sulfated, glycosylphosphatidylinositol (GPI)-linked extracellular proteins. The structural complexity of glypicans may underlie their functional complexity. In a recent study, we built on previous findings that one of the two C. elegans glypicans, LON-2, specifically inhibits signaling by the TGF-β superfamily member DBL-1. We tested the functional requirements of LON-2 protein core components and post-translational modifications for LON-2 activity. We provide the first evidence that two parts of a glypican can independently regulate TGF-β superfamily signaling in vivo: the N-terminal furin protease product and a C-terminal region containing heparan sulfate attachment sites. Furthermore, we show a protein-protein interaction motif is crucial for LON-2 activity in the N-terminal protein core, suggesting that LON-2 acts by serving as a scaffold for DBL-1 and an RGD-binding protein. In addition, we demonstrate specificity of glypican function by showing C. elegans GPN-1 does not functionally substitute for LON-2. This work reveals a molecular foundation for understanding the complexity and specificity of glypican function.

Introduction

Glypicans are complex proteins that play complex roles in the regulation of several intercellular signaling pathways, including transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), Hedgehog (Hh) and Wnt family members. Glypicans are composed of a cylindrical, α-helical protein core that is modified with heparan sulfate glycosaminoglycan (HS-GAG) chains and glycosylphosphatidylinositol (GPI), which anchors the glypican to the external surface of the cell membrane. Glypicans can be shed into the intercellular space through cleavage of the protein core at a furin cleavage site or by removal of the GPI lipid anchor. The interactions of glypican with cell-cell signaling factors is sometimes apparently contradictory and to correlate this structural complexity of glypicans with their function is a significant problem relevant to both developmental and disease biology. Both core protein and post-translational modifications differentially mediate glypican activity in animal developmental models and in cell lines (Table 1). Glypican-3 (GPC3), both membrane-linked and shed, is associated with a variety of cancer types and with cell growth. Clinical trials are evaluating the use of a monoclonal antibody against GPC3 to treat hepatocellular carcinoma (ClinicalTrials.gov). Parsing the roles of glypicans in specific cell signaling pathways is important to better understand the basis of developmental events and disease states affected by this proteoglycan family. Genetic interactions have been shown between glypicans and TGF-β members in...
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transgene product
sequence) and asked if transgene product
assay for activity of LON-2 variant trans -
grafting differentially contributes to Dally’s varied cellular roles through
regulation of TGF-β and other pathways
Table 1).4,50 No other study to date has
dissected the functional requirements for
the glypicain protein core or post-transla-
tional modifications in TGF-β superfam-
ily signaling (Table 1).
Recently, we systematically analyzed
these structural components of glypicain in regulating TGF-β signaling.31 We used the C. elegans glypican LON-2 because it
plays a clear role in specifically controlling
TGF-β signaling at the level of the TGF-β
ligand DBL-1 (Fig. 1A). This pathway regulates body length in a dose-dependent
manner, so we used body length as a bio-
assay for activity of LON-2 variant trans-
gene products. Loss of endogenous LON-2
function releases the “brakes” on DBL-1
body length-promoting activity, result-
ing in animals that are visibly longer than
normal.24 We introduced transgenes into
lon-2 mutant animals (caused by a dele-
tion that removes most of the lon-2 coding
sequence) and asked if transgene product
could restore a more normal body length.
We investigated the role of LON-2’s hepa-
ransulfate side chain and GPI attach-
We also narrowed the region
of the LON-2 core protein that regulates
TGF-β signaling and investigated the role
of a furin protease cleavage site and a
protein-protein interaction motif, RGD. We
discovered that membrane localization of
LON-2 is not required for its activity.
We have been able to uncover unexpected
functional requirements and redundancies
within the LON-2 structure related to its
function as a TGF-β signaling regulator.
LON-2 does not Require GAG
Attachment Sites or GPI Anchored
Membrane Attachment to Inhibit
DBL-1 Signaling
HS-GAG attachment sites are a charac-
teristic feature of all glypicains;16 however,
the functional requirement for glycosylation
varies from system to system (Table
1).1,2,4,7,9,11-13,21,30,32-34 GAG attachment sites
are not essential for the function of the
protein core of Drosophila Dally in wing
development or to bind TGF-β superfam-
ily members, but heparan sulfation of
Dally is required during eye development
and in vitro systems.4,50 For regulating
Hedgehog signaling, human GPC5
requires its heparan sulfate side chains,
but Dally-like, the other Drosophila
glypicain, does not.2,11,13,21,33 Similarly, dif-
ferent glypicans have different heparan
sulfation requirements to regulate Wnt
signaling.1,4,6,7,9,11,34-36 C. elegans glypican LON-2 has three
predicted sites for attachment of HS-GAG
chains (Fig. 1A). We found that lon-2
constructs with all three GAG attach-
ment sites altered could rescue the long
body size phenotype of animals lacking
endogenous LON-2, indicating that GAG
sites are not required for LON-2 to inhibit
DBL-1 signaling. Wild-type GFP-tagged
LON-2 visibly localizes to cell surfaces.24
LON-2 lacking all three GAG attachment
sites localizes to the cell surface similar to
the wild-type protein. This result shows
that the function and proper subcellular
localization of LON-2 is not dependent
on the sites for heparan sulfate side chain
attachment.
Another defining feature of glypi-
cans is their GPI glycolipid anchor,
which links the protein to the extracel-
ular membrane.77 Glypicans are shed
from their GPI membrane anchor during
development and disease states.36,38-42 In
Drosophila, substituting a transmem-
brane domain for the GPI linkage site still
allows Dally-like protein to inhibit Wg
signaling.41 However, Drosophila Dally-
like and mouse GPC3 require membrane
tethering by GPI to function in Hedgehog
signaling.2,21,35,43 Furthermore, a secreted
form of Drosophila Dally has a weak
dominant negative effect on signaling by
TGF-β superfamily member Dpp.43 We
found in C. elegans, the GPI-deleted form
of LON-2 is as functional as full-length
LON-2. This GFP-tagged construct fails
to visibly localize to cell membranes at
cell-cell junctions, an expected pheno-
type given its lack of membrane anchor.
Though extracellular LON-2 lacking a
GPI link was not visible, this LON-2 vari-
ant appeared as a bright haze within cells,
suggesting that this variant affects protein
secretion or stability. Our work indicates
that membrane localization by GPI is not
absolutely required for LON-2 to inhibit
DBL-1 activity.
The N-Terminal LON-2 Protease
Product can Inhibit DBL-1 Activity
At least one furin proprotein conver-
tase cleavage site is present or predicted in all glypicains.16,19,24 Inactivating
the Drosophila Dally-like furin cleavage site
does not alter the effect of Dally-like in
Hedgehog signaling.21 Protease processing
of GPC3 is required to repress both
canonical and noncanonical Wnt sign-
aling in cell lines and during zebrafish
development, but is not required to stimu-
late canonical Wnt signaling in hepato-
cellular carcinoma.79,94 GPC3 protease
fragments have been identified in the sera
of hepatocellular carcinoma patients, but
their activity is unknown.1,45 However, a
role for proteolyzed glypican in TGF-β
signaling has not been analyzed. To test
if cleavage is important to glypican func-
tion, we altered the LON-2 furin cleavage
site sequence RLGR to ALGA to prevent
furin cleavage at this site. We found
this LON-2 furin site variant restored the long
body phenotype. It could also properly
localize to cell membranes at cell-cell
junctions. Thus, processing at this furin
cleavage site is not necessary for LON-2
to function as a negative regulator of body
size.
We explored the importance of the
N-terminal furin protease product of
LON-2 in DBL-1 signaling. The furin
protease cleavage product of LON-2,
LON-2(1–368), is smaller than the
Table 1. Comparison of glypican family member structure-function analyses

| Pathway | Glypican | Model System | Protein core active | Heparan sulfated C-terminus sufficient? | RGD domain required? | Furin protease site required? | Heparan sulfate required? | GPI required? | Reference |
|---------|----------|--------------|---------------------|----------------------------------------|----------------------|-------------------------------|---------------------------|--------------|-----------|
| TGFβ | LON-2 | *C. elegans* | Yes | Yes | Yes | No | No | No | Taneja-Bageshwar and Gumienny, 2012 |
| Dally | Drosophila (in vivo, in vitro) | - | - | - | - | - | - | No | - | Kirkpatrick et al., 2006 |
| Dally | Drosophila cell line | - | - | - | - | - | - | Yes | - | Dejima et al., 2011 |
| FGF | OCI-5/ GPC3 | Cell lines | - | - | - | - | - | - | Yes | - | Song et al., 1997 |
| Wnt | Dally | Drosophila | - | - | - | - | - | - | Yes | - | Tsuda et al., 1999 |
| Dally | Drosophila | - | - | - | - | - | No | - | - | Kirkpatrick et al., 2006 |
| XGly4/ GPC4 | *Xenopus* | - | - | No | - | - | - | - | - | Ohkawara et al., 2003 |
| GPC3 | Cell lines | - | - | - | - | - | No | Yes | - | Capurro et al., 2005 |
| OCI-5/ GPC3 | Cell lines | - | - | - | - | - | No | - | - | Song et al., 2005 |
| Dally-like | Drosophila | - | - | - | - | - | - | Yes | - | Gallet et al., 2008 |
| Dally-like | Drosophila | - | - | - | - | - | No | No | - | Yan et al., 2009 |
| GPC1 | Chicken embryos | - | No | - | Not tested | - | Yes | - | - | Shiau et al., 2010 |
| GPC3 | HCC | - | Yes | - | - | - | - | Yes³ | - | Zittermann et al., 2010 |
| Hh | Dally | Drosophila | - | - | - | - | - | Yes | - | Takeo et al., 2005 |
| OCI-5/ GPC3 | Cell lines | - | No | - | - | - | No | Yes | - | Capurro et al., 2008 |
| Dally-like | Drosophila | - | - | - | - | - | - | Yes | - | Gallet et al., 2008 |
| Dally-like | Drosophila | - | - | - | - | - | No | No | - | Yan et al., 2009; Yan et al., 2010 |
| Dally-like | Drosophila | - | - | - | - | - | No | No | - | Williams et al., 2010 |
| GPC5 | Cell lines | - | - | - | - | - | Yes | - | - | Li et al., 2011 |
| Unknown | OCI-5/ GPC3 | Cell lines | - | - | - | - | - | No | Yes | - | Gonzalez et al., 1998 |
| GPC3 | Cell lines | - | Yes | - | - | - | - | - | - | De Cat et al., 2003 |

¹Cell lines are derived from mammalian sources. ²HCC, hepatocellular carcinoma. ³GPC3 promotes Wnt signaling and cell growth, but GPC3 lacking GPI (sGPC3) inhibits Wnt signaling and cell growth of some HCC cell lines.
non-GPI tethered form of LON-2 and lacks GAG attachment sites (Fig. 1B). This protein core fragment was functional in our bioassay, though it did not visibly associate with cell membranes.

We further found that by attaching the GPI anchor sequence to this rescuing N-terminal sequence, excluding the furin protease recognition site, the LON-2 minimal protein core does not need to be cleaved to be active. This GPI-anchored N-terminal fragment was weakly visible at the cell junctions but appeared to localize mainly in the cytoplasm. This result indicates that the N-terminal part of LON-2 is active in our bioassay, but it requires its full C-terminal protein core for normal levels of extracellular localization.

The LON-2 C-Terminus Can Independently Inhibit DBL-1 Signaling

The predicted C-terminal portion of LON-2 produced by the cleavage at the consensus furin protease site is much smaller than the N-terminus and contains the heparan sulfate attachment sites and the GPI linkage site. The glycanated C-terminal protease product of any glypicans has not been shown to contain glypican activity by itself.5 We made a construct, LON-2(423–508), that encodes the unstructured C-terminal furin protease product and tested its ability to inhibit DBL-1 activity (Fig. 1C). This 86 amino acid fragment, which contains two GAG attachment sites and the GPI attachment site, effectively inhibited DBL-1 activity in animals lacking functional LON-2. When GAG attachment sequences were altered in this construct, activity was lost. These results strongly support the model that the DBL-1-inhibitory function of LON-2(423–508) resides in the heparan sulfate chains attached to the LON-2 protein. Next, we asked if the GPI attachment site was required for activity of this glycanated C-terminal LON-2 fragment. Though the activity of the N-terminal LON-2 core protein does not depend on a membrane tether, activity of the C-terminal glycanated LON-2 fragment does depend on its GPI link to the outer cell membrane because C-terminal LON-2 constructs lacking the GPI anchor sequence fail to rescue the lon-2 mutant phenotype.

Glypicans Show Functional Specificity

We have previously shown that the Drosophila glypican Dally, which shares low sequence homology to LON-2, can functionally substitute for LON-2 in our C. elegans body length assay.24 Because TGF-β superfamily members bind heparin, and because the small heparan-sulfated C-terminal LON-2 fragment is sufficient to restore DBL-1 pathway inhibition, we asked if glypican inhibition of this heparin-binding cell-cell communication factor is specific or promiscuous. GPN-1 is the only other glypican in C. elegans. It is normally expressed at different times and locations than LON-2 and promotes some cell migrations, but does not affect body length.24,66 Structurally, GPN-1 shares the features common to all glypicans, but contains four heparan sulfate attachment sites, one more than LON-2 and has low sequence similarity to LON-2. We drove expression of GPN-1 from the lon-2 promoter in animals lacking lon-2 and discovered that GPN-1 did not inhibit the DBL-1-mediated body length defect. This result shows that LON-2 is not functionally replaceable by GPN-1 and supports a model whereby glypicans show specificity, possibly directed by the protein core, for binding heparan sulfate-binding ligand(s).

Soluble LON-2 Function Requires its RGD Tripeptide Motif or GPI Linkage

A tripeptide arginine-glycine-aspartic acid (RGD) motif can be found in many proteins of the extracellular matrix and regulates interactions between proteins and extracellular matrix constituents, particularly integrins.87–90 This sequence is shared within its family only by mammalian glypican-1 proteins, but it is common in many other extracellular TGF-β superfamily regulatory molecules. We previously showed that the RGD sequence is dispensable in the context of full-length, glycanated LON-2, but our recent work shows that heparan sulfation sites
can confer activity independent of the N-terminal protein core, which includes the RGD site.24 Therefore, we tested the requirement of the RGD sequence for the function of the N-terminal protein core. We showed that N-terminal LON-2 lacking the RGD site was expressed but was not functional in our bioassay. We then asked if RGD was required in the context of the non-glycosylated full length LON-2 variant. This variant lacking both RGD and GAG attachment sites is active, suppressing the DBL-1-mediated long body size defect of lon-2 mutant animals. This activity depends on the GPI anchor sequence, because modification of this construct to remove the GPI anchor sequence renders this variant inactive. These results show that when the full-length core protein is GPI linked to the membrane, the RGD motif is dispensable. Thus, RGD is not the sequence in the LON-2 core protein that directly regulates DBL-1 activity. Instead, this result indicates that the RGD binding to an unidentified protein facilitates DBL-1 inhibition by another part of the LON-2 N-terminus.

**Conclusion and Future Directions**

Glypicans play a complex role in regulating cell-to-cell signaling, but the mechanism of this regulation is not fully understood, especially for TGF-β superfamily signaling.5,16,51 By exploiting the long body phenotype of deregulated signaling by TGF-β superfamily member DDBL-1 in *C. elegans*, we showed that LON-2 activity resides in two separable regions (Fig. 1B and C). These regions have specific functional requirements. First, we identified the smallest functional glypican protein core region to date (LON-2(1–386)) and showed that it requires either its RGD protein-protein interaction motif or membrane association through GPI (Fig. 1B). Second, we discovered that the disordered C-terminal region is also bioactive and showed that this activity requires both heparan sulfate attachment sites and a GPI linkage sequence (Fig. 1C). Our work shows that soluble glypicans products are active in our system. Based on our studies and the works of others, we propose that glypicans in higher systems differentially regulate TGF-β and other signaling pathways in various tissues using both N- and C-terminal parts, membrane attached or shed.

Furthermore, these studies are the first to suggest the involvement of an additional player that interacts with the glypican protein core to control growth factor signaling. This result supports a model that LON-2 acts as a scaffold in a DBL-1-regulatory complex by binding both DBL-1 and an RGD-binding protein. LON-2 may act like the secreted glycoprotein fibrillin-1, an RGD-containing protein that regulates TGF-β signaling by scaffolding TGF-β and latent TGF-β binding protein (LTBP), integrins and other extracellular matrix proteins.32 It will be exciting to identify the LON-2-interacting protein. Determining if—and possibly how—other glypicans recruit accessory proteins to regulate signaling by TGF-βs and other pathways will be critical to understand glypican function and specificity during development and cancer progression.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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