Murine astrotactins 1 and 2 have a similar membrane topology and mature via endoproteolytic cleavage catalyzed by a signal peptidase

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Astrotactins are vertebrate-specific integral membrane glycoproteins known to play critical roles in central nervous system and skin development (1–4). An understanding of the function of Astn1 and Astn2 in the control of neuronal migration and synaptic function could be important for treatment of human brain disorders such as epilepsy and autism spectrum disorders. Although the number of gene mutations that can disrupt neuronal migration is large (5), Astn1 is one of a few adhesion receptors shown to directly function in migration (6).

In the mouse, there are two astrotactin family members, Astn1 and Astn2 (ASTN1 and ASTN2 in humans). Astn1 is involved in glial-guided neuronal migration early in development (1, 3, 6, 7) through the formation of an asymmetric complex with N-cadherin (CDH2) in the glial membrane (6). Astn2, which is 48% homologous to Astn1 and has two isoforms, is abundant in migrating cerebellar granule neurons, where it forms a complex with Astn1, and regulates the trafficking of Astn1 during migration (4). At later stages of development, Astn2 regulates synaptic function by trafficking of other membrane receptors, including Neuroligins and other synaptic proteins (8). A recent structure of the C-terminal endodomain of Astn2 shows distinctive features responsible for its activity (9). Astn1 and Astn2 are believed to share the same membrane topology, with a cleaved N-terminal signal peptide (SP), two transmembrane helices (TMHs), and a large extracellular C-terminal domain (10). Both Astn1 and Astn2 undergo an endoproteolytic maturation step in which an unknown protease cleaves the protein just after the second TM segment, with the two fragments remaining attached through a single disulfide bond (10, 11).

In this work, we mapped the topologies of mouse Astn1 and Astn2 in rough microsomal membranes using glycosylation mapping and protease protection assays. We found that Astn2 has a cleaved N-terminal SP, an N-terminal domain located in the lumen of the RM (topologically equivalent to the extracellular surface in cells), two TMHs, and a large extracellular C-terminal domain. We further show that Astn1 has the same topology as Astn2 but saw no evidence of SP cleavage for Astn1. Finally, we identify the endoprotease responsible for the maturation of Astn1 and Astn2 as the endoplasmic reticulum signal peptidase.

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This article contains Figs. S1 and S2.

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3 The abbreviations used are: Astn, Astrotactin; SP, signal peptidase; TMH, transmembrane helix; RM, rough microsome; ER, endoplasmic reticulum; OST, oligosaccharyl transferase; PK, proteinase K; Endo-H, endoglycosidase H; SPI, signal peptidase inhibitor.

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Results

Predicted topologies of mouse Astn1 and Astn2

Topology predictions for mouse Astn1 (UniProtKB Q61137-1, splicing isoform 1) and Astn2 (UniProtKB Q80Z10-3, splicing isoform 3) produced by the TOPCONS server (12) agree with the topology model for Astn2 derived from epitope tagging and cell surface staining (11); i.e., an N-terminal SP followed by two transmembrane segments (TMH1 and TMH2) and a large C-terminal extracellular domain (Fig. 1). In cells, both Astn1 and Astn2 are cleaved by an unidentified endoprotease into two fragments that remain linked by a disulfide bond (11). Edman sequencing of the two Astn2 fragments showed that the N-terminal one starts at Gly52 (just after the predicted signal peptide) and the C-terminal one at Asn466 (corresponding to Asn414 in the isoform analyzed here). For Astn1, the C-terminal fragment starts at Ser402; no sequence could be obtained from the N-terminal fragment in this case.

Topology mapping of mouse Astn1

To characterize the mouse Astn1 protein, we used a well-established in vitro glycosylation assay (13, 14) to determine the topology of the protein when cotranslationally inserted into dog pancreas rough microsomes (RMs). The transfer of oligosaccharides from the oligosaccharide transferase (OST) enzyme to natural or engineered acceptor sites for N-linked glycosylation (\(-NXS/TY\), where \(X\) and \(Y\) cannot be Pro (15–18)) in a nascent polypeptide chain is a characteristic protein modification that can only happen in the lumen of the ER, where the active site of the OST is located (19, 20). The topology of Astn1 in RMs was also probed by treatment with proteinase K, which can only digest parts of the protein protruding from the cytosolic side of the RMs (21).

To be able to investigate the topology of the 1302-residue-long and heavily glycosylated Astn1 protein, we selected to work with various truncated versions of the full-length protein. This was necessary because in vitro translation of such large proteins is inefficient and because attachment of an oligosaccharide increases the size of the protein by only 2–3 kDa, a shift that is too small to be detectable by SDS-PAGE for the full-length protein but can easily be visualized when using truncated versions.

Truncated versions of Astn1 were expressed in vitro using the TNT® SP6 Quick Coupled System supplemented with column-washed dog pancreas RMs (14, 21). The glycosylation status was investigated using SDS-PAGE, and truncated Astn1 versions were designed so that differences in glycosylation patterns could be used to infer the topology of the protein in the RM membrane.

Astn1 1–381, a version that extends from the putative SP to the end of the loop between TMH1 and TMH2, receives a single

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Figure 1. TOPCONS topology predictions. A, overview of the sequence of Astn1, with hydrophobic segments (blue), potential acceptor sites for N-linked glycosylation (\(\gamma\)), and proteolytic cleavage sites (red triangles) determined by Edman sequencing (11) marked. The TOPCONS topology prediction (http://topcons.cbr.su.se (34)) is given below. TOPCONS is a consensus predictor that collects data from the other prediction servers listed. B, the same for Astn2.
Topology and cleavage of astrotactins

glycan when translated in the presence of RMs (Fig. 2A, compare lanes 1 and 2). Notably, there is no sign of the SP being cleaved (which would reduce the molecular mass of the protein by 2.6 kDa). Astn1 78–381 (Fig. 2A, lanes 3 and 4) and Astn1 78–451 (Fig. 2A, lanes 5 and 6) also receive only a single glycan, whereas Astn1 78–470 (Fig. 2A, lanes 7 and 8) is glycosylated on two sites (note that glycan acceptor sites are rarely, if ever, modified to 100% in the in vitro translation system; hence, molecules with both one and two added glycans are visible on the gel). The second glycan addition, therefore, must be on Asn451.

To determine whether the first glycan addition is on Asn115 or Asn226 (Asn328 is too close to TMH2 to be reached by the protease), we expressed Astn1 versions lacking the entire N-terminal region, up to but not including TMH2 (Fig. 2B). The two shorter versions were not glycosylated at all when expressed in the presence of RMs, whereas Astn1 160–470 was modified on a single glycosylation site. The latter must be Asn451, showing that neither Asn226 nor Asn328 becomes glycosylated. We conclude that the putative SP in Astn1 appears not to be cleaved by signal peptidase and probably forms an N-terminal transmembrane helix (TMH0) and that Astn1 has two segments (residues 22–152 and 402–1302) exposed to the lumen of the RMs and one segment (residues 174–380) exposed to the cytosol. Further, because Asn115 is glycosylated in all four constructs, it appears that the N-terminal segment in the Astn1 constructs that start at Met78 can be translocated to the luminal side of the RMs even though it lacks the putative SP.

We further used a protease protection assay (21) to verify the proposed topology of Astn1. So that segments of Astn1 that are protected from proteinase digestion by the RM membrane would be of a convenient size for SDS-PAGE separation, we first expressed Astn1 78–728. The protein becomes glycosylated (Fig. 2C, compare lanes 1 and 2), but it is difficult to determine on how many sites. Interestingly, two prominent bands at ~38 kDa (marked N) and ~36 kDa (marked C) were generated in the presence of RMs (Fig. 2C, lane 2), suggesting internal endoproteolytic cleavage, in agreement with the published Edman sequencing results that identified a cleavage site between Ser401 and Ser402 (11). In addition, a third band at ~65 kDa that appears to receive a single glycan in the presence of RMs was also seen (Fig. 2C, lanes 1 and 2). The latter would be consistent with internal translation initiation at Met460 and indeed comigrates with Astn1 160–728 (Fig. 2C, lane 4).

Proteinase K treatment of RMs carrying Astn1 78–728 digests cytoplasmically accessible parts of the protein and leaves only two protected fragments: one of identical size to the “endoproteolytic” 36-kDa band and one at ~39 kDa (Fig. 2C, lane 3). The two protease-protected fragments are precisely what would be expected from the topology derived from the glycosylation study; the 39-kDa band (marked C′) represents fragment 381–728, generated when proteinase K digests the cytosolic loop, and the 36-kDa band represents the slightly smaller C-terminal fragment 402–728, generated by endoproteolytic cleavage near the C-terminal end of TMH2. The expected protected N-terminal fragment 78–181 is too small to be resolved on the gel.

Similar results were obtained for Astn1 160–728. In addition to the full-length protein at ~65 kDa, two bands at ~36 kDa (marked C) and ~25 kDa (marked N) were seen in the presence of RMs (Fig. 2C, compare lanes 5 and 6); Endo-H treatment shifted both the full-length band at ~65 kDa and the ~36 kDa band to a lower molecular mass, whereas the 25-kDa band did not shift (Fig. 2C, lane 8). Consistent with the Astn1 160–728 results, the glycosylated, ~36-kDa band represents the same endoproteolytic C-terminal fragment 402–728, whereas the unglycosylated 25-kDa band represents the N-terminal endoproteolytic fragment 160–401.

Given the sequence context of the endoproteolytic cleavage site (see “Discussion”), we hypothesized that the responsible protease may be a signal peptidase. Indeed, inclusion of a signal peptidase inhibitor (23) in the in vitro translation of Astn1 160–728 completely inhibits formation of the ~36-kDa and ~25-kDa products (Fig. 2C, lane 11).

We conclude that Astn1 has the same topology as proposed previously for Astn2; namely, with two lumenal domains (residues 22–152 and 173–1302) and one cytosolic domain (residues 174–381). The putative SP appears not to be cleaved but, rather, forms an N-terminal transmembrane helix (TMH0). We identify signal peptidase as the enzyme responsible for the endoproteolytic cleavage event at Ser401.

Topology mapping of mouse Astn2

We used the same glycosylation mapping approach to determine the topology of the 1300-amino-acid-long mouse Astn2 protein (splice isofrom 3, lacking exon 4, which encodes a 52-residue segment in the domain between TMH1 and TMH2). Astn2 1–482 includes both the putative SP, the two predicted transmembrane helices TMH1 and TMH2, and a portion of the large C-terminal domain. A small amount of glycosylated full-length product at ~56 kDa, two weak bands at ~50 kDa that might represent glycosylated and unglycosylated products lacking the SP (which has a calculated molecular mass of 6.4 kDa), and a prominent product at ~43 kDa are seen in the presence of RMs (Fig. 3A, lanes 2, 4, and 5). The latter is sensitive to Endo-H digestion, and the two bands at ~50 kDa collapse to the lower-molecular-mass form upon the same treatment (Fig. 3A, lane 6). The glycosylated 43-kDa band fits the molecular mass expected for a product resulting from removal of the signal peptide (residues 1–51) and the endoproteolytic cleavage at Asn413 observed by Edman sequencing (11) (note that we use a different splice version of Astn2 that lacks 52 residues in the cytosolic segment compared with the one used in this reference). This explains the limited amount of glycosylated full-length product (Fig. 3A, lanes 2, 4, and 5) because most of the molecules that become glycosylated are cleaved after the SP and/or TMH2, as seen in Fig. 3A, lane 6.

To confirm this interpretation, we also analyzed Astn2 161–482, which lacks the putative SP. Astn2 161–482 yields four prominent bands when expressed in the presence of RMs (Fig. 3B, lane 2); unglycosylated full-length product at ~37 kDa, singly and doubly glycosylated full-length products at ~39 kDa and ~42 kDa, and a smaller endoproteolytic product at ~35 kDa. Endo-H treatment collapses the ~39-kDa and ~42-kDa bands to the size of the unmodified full-length product at ~37
Figure 2. Topology mapping of Astn1 and inhibition of endoproteolytic cleavage by an inhibitor of signal peptidase. A, the indicated truncated versions of Astn1 were translated in vitro with [35S]Met in the presence (+) or absence (−) of RMs and analyzed under reducing conditions by SDS-PAGE. Unglycosylated products are indicated by an open circle, singly glycosylated products by a filled circle, and doubly glycosylated products by two filled circles. The glycosylated Asn residues are indicated by a red circle in the cartoon. Mw, molecular weight. B, the same as in A. C, Astn1 78–728 was translated in vitro with [35S]Met with or without RMs (lanes 1 and 2). RMs were subjected to PK digestion (lane 3). The N- and C-terminal fragments resulting from endoproteolytic cleavage between Ser401 and Ser402 are indicated (N and C, respectively), as is the protease-protected C-terminal fragment (C*). RMs carrying Astn1 160–728 were subjected to Endo-H (EH) digestion (lanes 4–8). Note the shift in mobility for the full-length and C bands caused by deglycosylation (compare lanes 7 and 8). Astn1 160–728 was also translated in vitro with [35S]Met in the presence (+) or absence (−) of RMs and the signal peptidase inhibitor SPI (lanes 9–11).
kDa and the ~35-kDa band to a smaller, ~30-kDa band (Fig. 3B, lane 5). Similar to Astn1, addition of the signal peptidase inhibitor to the in vitro translation completely inhibits formation of the ~35-kDa endoproteolytic product (Fig. 3B, lane 3), and the signal peptidase inhibitor plus Endo-H treatment of RM-integrated Astn2 161–482 leaves only the unmodified full-length product (Fig. 3B, lane 7; for unknown reasons, the signal peptidase inhibitor makes bands run slightly higher in the gel).
These results are entirely consistent with the proposed topology of Astn2 (11) and identify signal peptidase as the enzyme responsible for the endoproteolytic cleavage event at Asn413.

**Discussion**

Earlier work using epitope mapping of Astn2 expressed in COS7 cells has shown that the N and C termini are exposed on the cell surface, whereas the domain between TMH1 and TMH2 can only be immunodecorated in detergent-permeabilized cells (11). Further, both Astn1 and Astn2 have been shown to be cleaved by an unknown endoprotease into an N- and a C-terminal fragment, and Edman sequencing of the C-terminal fragments identified cleavage sites between Ser401-Ser402 in Astn1 and Gly465-Asn466 in Astn2, just after TMH2. In addition, for Astn2, Edman sequencing of the N-terminal endoproteolytic fragment indicated removal of the putative SP (residues 1–51); no sequence was obtained for Astn1, leaving open whether the putative SP is cleaved in this protein.

Here we confirmed and extended these results for Astn1 and Astn2 using glycosylation mapping and protease protection assays in a coupled *in vitro* transcription–translation system supplemented with RMs. Our results for Astn2 are in perfect agreement with those from the earlier study; Astn2 has a cleaved N-terminal SP, an N-terminal domain located in the lumen of the RM (topologically equivalent to the extracellular surface in cells), two TMHs, and a large C-terminal lumenal domain (Fig. 4). We found that Astn1 has the same topology as Astn2 but saw no evidence of SP cleavage; rather, it seems that the putative N-terminal SP in Astn1 remains a part of the protein, presumably forming a third transmembrane helix (TMH0).

We further show that an inhibitor of the signal peptidase complex completely inhibits the endoproteolytic cleavage of both Astn1 and Astn2. The unknown endoprotease involved in the maturation of Astn1 and Astn2 is thus signal peptidase, the enzyme that cleaves SPs from secretory and membrane proteins in the ER (24). Although it is uncommon that signal peptidase catalyzes internal cleavage reactions of this kind in cellular proteins, many viral polyproteins mature through signal peptidase–catalyzed cleavages after internal hydrophobic segments in the primary translation product (25, 26). Indeed, the SP cleavage site and the cleavage site after TMH2 identified by Edman sequencing in Astn2 are precisely the ones predicted by the SignalP server (27) (Fig. S2).

These findings raise the possibility that higher levels of SP-mediated cleavage of Astn2 relative to Astn1 explain the higher levels of the Astn1 C terminus we detected previously on central nervous system neuronal surface membranes by antibody labeling and functional assays (6, 8). This also likely contributes to the apparently distinct functions of Astn1 as a membrane adhesion receptor that functions in glial-guided migration (3, 6, 7) and of Astn2 as an endolysosomal trafficking protein that functions in both migration (4) and synaptic function (8). Finally, the exceptionally long Astn2 SP hints at the possibility that, after cleavage, the SP may have additional functions in the cell, as seen for many other very long SPs (28). It will therefore be of interest to determine whether the Astn2 SP domain functions in receptor trafficking or planar polarity signaling pathways.

**Experimental procedures**

**Enzymes and chemicals**

Unless otherwise stated, all chemicals were from Sigma-Aldrich (St. Louis, MO). Plasmid pGEM1, the TNT® Quick Coupled Transcription/Translation System, the rabbit reticulocyte lysate system, and deoxynucleotides were from Promega (Madison, WI). [35S]Met was from PerkinElmer Life Sciences. All enzymes were from Fermentas (Burlington, ON, Canada), except Phusion DNA polymerase, which was from Finnzymes (Espoo, Finland), and SP6 RNA polymerase, which was from Promega. The QuikChange™ site-directed mutagenesis kit was from Stratagene (La Jolla, CA), and oligonucleotides were from Eurofins MWG Operon (Ebersberg, Germany). All other reagents were of analytical grade and were obtained from Merck (Darmstadt, Germany).
DNA manipulation

The complementary DNAs of mouse Astn1 and Astn2 (1302 and 1300 amino acid residues, respectively; Fig. S1) were cloned into the pR5K vector using Clal/Sall (Astn1) and BamHI/XbaI (Astn2) sites. The DNA was then transferred to the pGEMI vector (Promega) at XbaI/SmaI sites together with a preceding Kozak sequence (29), as described previously (13). To create truncations in Astn1, deletions were made between amino acid position 1–78 and 1–160, and stop codons were introduced at positions 382, 452, 471, and 729. Astn2 truncations were created in the same way, with a deletion between 1–161 and a stop codon at 483. The Astn1 and Astn2 cDNAs were amplified by PCR using the Phusion DNA polymerase with appropriate primers, and site-specific mutagenesis was performed using the QuickChange™ site-directed mutagenesis kit from Stratagene. All mutants were confirmed by sequencing of plasmid DNA at Eurofins MWG Operon and BM Labbet AB (Furu-lund, Sweden).

In vitro expression

All Astn constructs cloned in pGEMI and pRK5 were transcribed and translated in an in vitro the Tnt™ SP6 Quick Coupled System from Promega. 150–200 ng DNA template, 1 μl of [35S]-Met (5 μCi) and 0.5 μl column-washed dog pancreas rough microsomes (RM) (tRNA Probes, US) (30) were added to 10 μl of reticulocyte lysate at the start of the reaction, and the samples were incubated for 90 min at 30 °C (21).

Proteinase K treatment

PK treatment was performed by adding 1 μl of CaCl2 (200 mm) and 0.2 μl of Proteinase K (4.5 units/μl) to the translation reaction. After incubation on ice for 30 min, 1 ml of PMSF (20 mm ethanolic solution) was added to inactivate PK, and samples were further incubated on ice for 5 min (21).

Endo-H treatment

For endoglycosidase H (Endo-H) treatment, 9 μl of the Tnt reaction product was mixed with 1 μl of 10× glycoprotein denaturing buffer. Following addition of 1 μl of Endo-H (500,000 units/ml; New England Biolabs), 7 μl of distilled H2O and 2 μl of 10× G3 reaction buffer, and the sample was incubated at 37 °C for 1 h (31). Mock controls were identical but lacked Endo-H.

SPL treatment

To demonstrate cleavage by signal peptidase, the inhibitor SPL (N-methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone, Sigma) was dissolved in dimethyl sulfoxide (DMSO) and added to the translation mixture at a final concentration of 1.4 mm as described previously (14, 23, 31–33).

Analysis and quantitation

Translation products were analyzed under reducing conditions by SDS-PAGE, and proteins were visualized in a Fuji FLA 9000 Phosphorimager (Fujiﬁlm, Tokyo, Japan) using the Image Reader FLA 9000/Image Gauge V 4.23 software (Fujiﬁlm).

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