The Paranodal Complex of F3/Contactin and Caspr/Paranodin Traffics to the Cell Surface via a Non-conventional Pathway*

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During myelination, membrane-specialized domains are generated by complex interactions between axon and glial cells. The cell adhesion molecules caspr/paranodin and F3/contactin play a crucial role in the generation of functional septate-like junctions at paranodes. We have previously demonstrated that association with the glycosylphosphatidylinositol-linked F3/contactin is required for the recruitment of caspr/paranodin into the lipid rafts and its targeting to the cell surface. When transfected alone in neuroblastoma N2a cells, caspr/paranodin is retained in the endoplasmic reticulum (ER). Using chimerical constructs, we show that the cytoplasmic region does not contain any ER retention signal, whereas the ectodomain plays a crucial role in caspr/paranodin trafficking. A series of truncations encompassing the extracellular region of caspr/paranodin was unable to abolish ER retention. We show that N-glycosylation and quality control by the lectin-chaperone calnexin are required for the cell surface delivery of caspr/paranodin. Cell surface transport of F3/contactin and caspr/paranodin is insensitive to brefeldin A and the two glycoproteins are endoglycosidase H-sensitive when associated in complex, recruited into the lipid rafts, and expressed on the cell surface. Our results indicate a Golgi-independent pathway for the paranodal cell adhesion complex that may be implicated in the segregation of axonal subdomains.

Myelination is of key importance in vertebrates, ensuring rapid saltatory conduction of nerve impulses. The nodes of Ranvier that contain high density of voltage-gated sodium channels are flanked by the paranodes where the terminal loops of myelinating glial cells are sealed to the axonal membrane through septate-like junctions. The juxta-paranodal regions highly enriched in potassium channels are bordering the paranodes. Contacts with myelinating glial cells generate specialized axonal domains in the region of the nodes of Ranvier, with highly specific composition in ion channels, cell adhesion molecules (CAMs),¹ and cytoskeletal linker protein (1, 2). The axonal subdomains are progressively established during development or remyelination events, by the limitation of lateral diffusion of membrane components and their clustering, mediated by CAMs and scaffolding elements (3–6). However, additional mechanisms may operate such as selective trafficking and regulated endocytosis (7) that should be especially crucial for the renewal of membrane components in each subdomain.

A complex of CAMs have been identified at paranodes comprising caspr (contactin-associated protein)/paranodin and F3/contactin that are associated on the axolemma and bind the glial component neurofascin-155 (8, 9). F3/contactin is a glycosylphosphatidylinositol-anchored Ig-CAM that is implicated in multiple heterophilic binding (10, 11). Caspr/paranodin is a transmembrane molecule of the neurexin superfamily with an extracellular region bearing a N-terminal discoidin domain, several laminin-G and EGF-like regions, and a fibrinogen central domain (12, 13). The intracellular region of caspr/paranodin contains a juxta-membrane region for the binding of FERM (four-point-one, ezrin, radixin, moesin) molecules, including 4.1B and schwannomin (14–16). A recent study shows that the extracellular region of caspr/paranodin is sufficient to direct it to paranodes in transgenic mice (14). However, the cytoplasmic domain is required for the stabilization of caspr/paranodin at the paranodal membrane most likely through the linkage with the cytoskeleton-associated protein 4.1B.

Deficiency in either caspr/paranodin or F3/contactin in the respective knockout mice results in the aberrant organization of the paranodal region, disruption of septate junctions, and reduction of nerve conduction velocity (17, 18). The fence function of septate junctions has been well evidenced from the mutant phenotype, because their disruption results in the lateral shift of the potassium channels that are mislocalized at paranodes instead of juxtaparanodes. In addition, mutant analyses show that caspr/paranodin and F3/contactin are interdependent for their paranodal distribution. Interestingly, two distinct forms of F3/contactin are expressed at the nodes and paranodes, respectively, which differ in their N-glycan content (4). In caspr/paranodin-deficient mice, F3/contactin is not found at paranodes, but is enriched in the nodal region (17). Caspr/paranodin and F3/contactin assemble early during biosynthesis in the ER and this association is required for an efficient targeting of caspr/paranodin to the surface of transplanted cells (19). In agreement with the in vitro analysis, caspr/paranodin expression is restricted to the neuronal cell bodies

contactin-associated protein; EGF, epidermal growth factor; ER, endoplasmic reticulum; Endo H, endoglycosidase H; GFP, green fluorescent protein; aa, amino acids; CHO, Chinese hamster ovary; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; mAb, monoclonal antibody; PNGase F, N-glycosidase F.
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and not addressed to the axolem in F3/contactin-deficient mice (18).

In the present study, we have investigated the mechanisms involved in caspr/paranodin and F3/contactin trafficking to the cell surface. The intracellular transport of glycoproteins from the ER to the cell surface is a tightly controlled process that allows quality control, regulation of subunit assembly, and sorting of proteins to specific destinations. Different mechanisms such as masking and unmasking of retention signals, association with ER chaperones, or thiol-mediated retention happen in the ER (50). In addition, it is now well known that N-linked glycans play a pivotal role in protein folding, quality control, sorting, and transport of glycoproteins (21–23). Here we show that caspr/paranodin cell surface targeting depends on N-glycosylation and on quality control by the lectin-chaperone calnexin. In addition, we present evidence that the complex of caspr/paranodin and F3/contactin reaches the plasma membrane with high-mannose type N-glycans, via an unconventional pathway insensitive to brefeldin A, as recently described for CD45 (24) or for the flotillin-1-reggie-2 raft component (25). Our data indicate that F3/contactin traffics to the cell surface via two distinct routes, one involving the Golgi complex, resulting in endoglycosidase H (Endo H)-resistant carbohydrate processing, and the other independent of the Golgi, resulting in the maintenance of Endo H-sensitive carbohydrate when associated with caspr/paranodin on the cell surface.

EXPERIMENTAL PROCEDURES

Antibodies—We used a rabbit antiserum prepared against the F3/contactin Ig-like domains and designated 24 for immunofluorescence staining (26) and a rabbit antiserum directed against amino acids (aa) 37–50 of F3/contactin for immunoblotting (27). Rabbit antiserum SL51, which was raised against a glutathione S-transferase fusion protein encompassing residues 1303–1380 of caspr/paranodin, reacted with epitopes in its intracellular region (12). The anti-caspr2 antibody was generated by immunizing rabbits with the intracellular region (residues 1284–1331) fused to glutathione S-transferase. Human CD4 cDNA and mouse anti-CD4 mAb were donated by J. Merot (Centre d’Etude Atomique, Saclay, France), mouse anti-GFP mAb was purchased from Roche Diagnostics, rabbit anti-calnexin antiserum was from StressGen Biotechnologies, Alexa 488-conjugated secondary antibodies were purchased from Molecular Probes. Peroxidase- and Texas Red-conjugated secondary antibodies were from Jackson ImmunoResearch.

Cloning Strategies—The DNA construct pBS-CMV/F3 encoding the full-length sequence of F3/contactin was previously described (28). The caspr/paranodin full-length sequence of F3/contactin (aa 1–1297) using the forward primer, 5’-GGAATTCGCAGATAGAAGAGCACCAGCAT-3’ and reverse primer, 5’-GAATTCCTGAAGGCTCCTGACCTGCTCTC-3’ was amplified with caspr/paranodin on the cell surface.

Cell Culture—CHO, COS-7, and neuroblastoma N2a cells were grown in DMEM containing 10% FCS. CHO cell lines stably transfected with caspr/paranodin or with F3/contactin, or coexpressing caspr/paranodin and F3/contactin were previously described (19). COS-7 and N2a cells were transiently transfected using LipofectAMINE Plus (Invitrogen). Single or double transfected N2a cells were cultured for 18 h in Opti-MEM (Invitrogen) before treatment with 2 µg/ml tunicamycin (Sigma), 2.5 µg/ml brefeldin A (Sigma), or 1 µg/ml caspase inhibitor (Sigma) for a maximum of 24 h.

Immunoprecipitation, Endoglycosidase Treatments, and Cell Surface Biotinylation—Transfected COS-7, N2a, and CHO cells were lysed for 30 min on ice with 50 mM Tris, pH 7.5, 1% Nonidet P-40, 10 mM MgCl2, and protease inhibitors, centrifuged at 4°C for 10 min, rinsed with PBS, and incubated for 1 h at 4°C with protein A-Sepharose. Supernatants were immunoprecipitated overnight at 4°C with protein A-Sepharose coated with SL51 antibody (3 µl), anti-GFP antibody (3 µl), or anti-caspr2 antibody (3 µl). The beads were washed twice with 50 mM Tris, pH 7.4, 150 mM NaCl, and 1% Nonidet P-40, twice in 50 mM Tris, 150 mM NaCl and twice in 50 mM Tris. Immune precipitates were analyzed by immunoblotting with anti-caspr/paranodin, anti-F3/contactin, anti-GFP, anti-caspr2, or anti-calnexin. Blots were developed using the ECL chemiluminescent detection system (Roche).

Low density Triton X-100-insoluble lipid rafts were isolated as previously described from double transfected CHO cells (19). CHO or N2a cells were solubilized and lipid raft fractions were adjusted to 1% 2-mercaptoethanol, 0.2% SDS, incubated with 3 milliunits of Endo H (Roche) or 1 unit of N-glycosidase F (PNGase F) (Roche) for 3 h at 37°C, and analyzed by immunoblotting with anti-caspr/paranodin or anti-F3/contactin.

Double transfected CHO cells were washed three times for 10 min with PBS at 4°C and then biotinylated with 0.5 mg/ml sulfo-NHS-LC biotin (Pierce) in 10 mM triethanolamine, pH 9, 140 mM NaCl for 20 min at 4°C. After two washes in PBS at 4°C, cells were lysed in 50 mM Tris, pH 7.4, 1% bovine serum albumin, 1% Nonidet P-40, and protease inhibitors. Immunoprecipitation of caspr/paranodin was carried out as described above, and elution was performed with 50 mM Tris, pH 7.5, containing 1% Nonidet P-40, 0.2% SDS, 1% 2-mercaptoethanol, and protease inhibitors. The immune precipitates were incubated with 3 milliunits of Endo H for 3 h at 37°C and analyzed by immunoblotting with anti-caspr/paranodin antibody or peroxidase-conjugated streptavidin.

Metabolic Labeling—Double transfected CHO cells were treated with 5 mM sodium butyrate during 18 h, incubated in Opti-MEM with or without 2 µg/ml tunicamycin for 2 h and then pulsed with 10 µCi/ml [35S]Met + Cys (Expre’SS–SS-label, Amersham Biosciences) for 1 h in DMEM without cysteine and methionine, supplemented with 10% FCS. After washing twice with PBS, cells were chased in DMEM, 10% FCS, for 2, 6, and 16 h. Immunoprecipitation with anti-paranodin antibody and SDS-PAGE analysis were performed as described above. The radioactivity was digitalized and quantified using a Molecular Imager GS363 (Bio-Rad).

Brain Extracts—Brain homogenate was in a buffer (6 ml/g) containing 50 mM Tris, 3 mM MgCl2, 320 mM sucrose, and protease inhibitors, using a Heidolph homogenizer. The homogenate was centrifuged at 1,000 × g for 15 min at 4°C. The supernatant was further centrifuged at 100,000 × g at 4°C for 1 h. The pellet was extracted in a buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 0.5% SDS, 1 mM EDTA, 0.05% sodium deoxycholate, 1% Triton X-100, 1% 2-mercaptoethanol, and proteases inhibitors. Samples were then treated with 3 milliunits of Endo H for 3 h at 37°C, separated by SDS-PAGE (7.5%), and analyzed by immunoblotting with anti-paranodin and anti-F3/contactin antibodies.

Immunofluorescence and Confocal Microscopy—N2a cells were plated on glass coverslips and transfected with different constructs. For visualization of CD4-pdF, N2a-transfected cells were fixed with 4% paraformaldehyde in PBS for 10 min and permeabilized with methanol for 10 min at −20°C, rehydrated in PBS, and incubated for 1 h with antibodies, conjugated to species-specific secondary antibodies labeled with Alexa 488 and 594. Cells were then permeabilized with 0.2% Triton X-100 for 5 min and washed with PBS for 10 min. After washing in PBS, cells were mounted in Mowiol (Calbiochem) and viewed on a Leica laser scanning confocal microscope. Expression of caspr2-, caspr/paranodin-, or paranodin-deleted constructs were double transfected cells after fixation with methanol for 10 min at −20°C, and immunofluorescence staining with SL51 or anti-caspr2 (1:2000) antibody. Pnd-GFP was detected by immunofluorescence staining with anti-GFP (1:250) in cells fixed with 4% paraformaldehyde in PBS for 10 min.
CD4 truncated
CD4-pnd

pnd
pnd-GFP
pnd-Δ1
pnd-Δ2
pnd-Δ3
pnd-Δ4
pnd-Δ5

Factor VIII/discoidin  Laminin G
Fibrinogen  PGY repeats
Signal peptide  EGF

Fig. 1. Schematic representation of the different expression constructs. A truncated form of CD4 that lacks the intracytoplasmic region of CD4 (CD4-truncated) was used as control. CD4-paranodin (CD4-pnd) contains the extracellular and transmembrane domains of CD4 fused with the complete cytoplasmic region of caspr/paranodin. Paranodin-GFP (pnd-GFP) is composed of the extracellular and transmembrane domains of caspr/paranodin-(1–1297) fused with GFP. Pnd-Δ1 is deleted from the discoidin to the fibrinogen domain (aa 22–781), pnd-Δ2 from the first laminin G to the fibrinogen domain (aa 186–781), pnd-Δ3 from the first EGF to the fibrinogen domain (aa 532–781), pnd-Δ4 from the third laminin-G domain to the juxtamembrane region (aa 781–1277), and pnd-Δ5 from the third laminin-G to the PGY domain (aa 781–1097).

RESULTS

The Cytoplasmic Region of Caspr/Paranodin Does Not Confer ER Retention—Caspr/paranodin is blocked in the ER when transfected alone in neuroblastoma N2a cells, whereas it is efficiently targeted to the cell surface when co-transfected with F3/contactin (19). However, the cytoplasmic region of caspr/paranodin does not appear to contain any consensus sequence for ER retention, such as the dibasic motifs operating in ER resident molecules (29). We tested whether the cytoplasmic region of caspr/paranodin is able to confer ER retention to the transmembrane reporter CD4, a T-cell surface protein. A CD4-pnd chimera was engineered by fusing the cytoplasmic region of caspr/paranodin to the extracellular and transmembrane domains of CD4 (Fig. 1). The CD4-pnd chimera was transfected in N2a cells. Double immunostaining with anti-CD4 mAb and an antiserum directed against the intracellular region of caspr/paranodin (SL51) shows that CD4-pnd is highly expressed at the cell surface and not detected in intracellular compartments (Fig. 2). This result indicates that the intracellular region of caspr/paranodin does not confer ER retention.

The Ectodomain Plays a Crucial Role in Caspr/Paranodin Trafficking to the Cell Surface—The role of the extracellular region of caspr/paranodin was then evaluated. We first generated a pnd-GFP chimera by fusing the extracellular and transmembrane domains of caspr/paranodin with GFP as a cytoplasmic tail, using the pEGFP-N1 vector (Fig. 1). Caspr/paranodin and pnd-GFP were both retained in the ER when transfected alone in N2a cells (Fig. 3, A and B). In contrast, both proteins were strongly expressed on the cell surface when co-transfected with F3/contactin (Fig. 3, C and D). F3/contactin is expressed as a doublet of 142 and 135 kDa in transfected N2a cells. Using immunoprecipitation with anti-GFP mAb and immunoblot for F3/contactin, we further demonstrated that pnd-GFP, like native caspr/paranodin, interacted with the 135-kDa form of F3/contactin (Fig. 3E). These data show that the extracellular region is necessary and sufficient for ER retention of caspr/paranodin and for the F3/contactin-mediated export to the cell surface.

As a second step, we generated constructs with large deletions all along the ectodomain of caspr/paranodin to map the domains implicated in ER retention and/or F3/contactin binding (Fig. 1). To minimize conformational alterations, the different constructs were designed with deletion sites at the border between structural modules. The deleted constructs were expressed in COS-7 cells and immunoprecipitated from Nonidet P-40 extracts with an anti-paranodin antibody directed against the cytoplasmic region. All the constructs were detected on immunoblots at the expected molecular weight (Fig. 4A). The immune precipitates from double transfected cells were analyzed by immunoblotting with anti-F3/contactin antibody. F3/contactin was strongly co-immunoprecipitated with caspr/paranodin in control experiments, whereas it was not precipitated with any of the deleted constructs (Fig. 4B).

Then, we investigated the consequence of the various truncations on caspr/paranodin trafficking in transfected N2a cells. Confocal analysis indicates that all the deleted constructs are distributed in the ER, without any cell surface expression (Fig. 4, D–H). N2a cells were co-transfected with F3/contactin and each of the deleted constructs. All types of deletion abolished the interaction with F3/contactin, and as a consequence, prevented F3/contactin-mediated cell surface targeting of the different constructs (not shown). The pnd-Δ1 and pnd-Δ4 constructs comprised deletions of the N- or C-terminal half of the ectodomain, respectively. These two constructs were retained in the ER indicating that caspr/paranodin does not contain an ER retention motif corresponding to a single sequence of amino acids, and may contain several motifs of retention. Alterna-
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FIG. 3. The ectodomain of caspr/paranodin is critically required for the control of ER exit. A–D, confocal analysis of caspr/paranodin and pnd-GFP expression in N2a cells. N2a cells were transiently transfected with caspr/paranodin (A), pnd-GFP (B), double transfected with caspr/paranodin and F3/contactin (C), or pnd-GFP and F3/contactin (D). After cell fixation and permeabilization, caspr/paranodin was detected using anti-paranodin antiserum (A and C) and pnd-GFP using anti-GFP mAb (B and D). When expressed alone, caspr/paranodin and pnd-GFP are retained in the ER (A and B). In contrast, when co-transfected with F3/contactin, caspr/paranodin and pnd-GFP are only detected at the cell surface (C and D). Confocal images were scanned using a Leica confocal microscope. Bar, 10 μm. E, coimmunoprecipitation of F3/contactin with caspr/paranodin or pnd-GFP from double transfected COS-7 cells. Cells were co-transfected with caspr/paranodin and F3/contactin (lanes 1, 2, 5, and 6) or with pnd-GFP and F3/contactin (lanes 3, 4, and 7). Cells were lysed 48 h after transfection using Nonidet P-40 and immunoprecipitation (Ip) was performed with anti-paranodin antiserum (lanes 2 and 6) or anti-GFP mAb (lanes 4 and 7). Caspr/paranodin (lane 1) and pnd-GFP (lane 5) are detected in the lysates (L) at the expected molecular masses of 190 and 210 kDa, respectively. F3/contactin is detected in the lysate (L) as a doublet corresponding to the glycosylation variants of 142 and 135 kDa (lane 5). The 135-kDa form of F3/contactin is co-immunoprecipitated with caspr/paranodin (lane 6) and pnd-GFP (lane 7). Experiments were made in triplicate. Wb, Western blot.

Fig. 4. The various deletions generated along the ectodomain of caspr/paranodin abolish interaction with F3/contactin and do not prevent ER retention. A and B, COS-7 cells were double transfected with F3/contactin and full-length caspr/paranodin (lane 1) or caspr/paranodin-deleted constructs (lanes 2–6). After cell lysis with Nonidet P-40, immunoprecipitation (Ip) with anti-paranodin antiserum was realized. Immunoblotting with anti-paranodin antiserum (A) shows that the different constructs are detected at the expected molecular mass, pnd-Δ1 (97 kDa), pnd-Δ2 (114 kDa), pnd-Δ3 (152 kDa), pnd-Δ4 (125 kDa), and pnd-Δ5 (145 kDa). B, F3/contactin is co-immunoprecipitated with full-length caspr/paranodin (lane 1), whereas it is not co-immunoprecipitated with the deleted constructs (lanes 2–6). The molecular mass markers are indicated on the left. Experiments were made in triplicate. C–H, expression of caspr/paranodin and deleted constructs in N2a cells as analyzed by confocal microscopy. Cells were transfected with caspr/paranodin (C), pnd-Δ1 (D), pnd-Δ2 (E), pnd-Δ3 (F), pnd-Δ4 (G), or pnd-Δ5 (H). Immunofluorescence staining with anti-paranodin antiserum indicates that all the deleted constructs are retained in the ER. Bar, 10 μm.

delivery of unglycosylated mutant Shaker is at least partially because of slowed export from the ER (31). Therefore, we examined the possible role of N-glycans in caspr/paranodin transport to the cell surface. N2a cells were transiently transfected with pnd-GFP alone or in association with F3/contactin. 18 h after transfection, cells were treated during 24 h with tunicamycin, an antibiotic that specifically prevents the early step of N-glycosylations (21). Tunicamycin treatment prevented the cell surface expression of pnd-GFP when co-transfected with F3/contactin in N2a cells (Fig. 5C). In double transfected N2a cells, co-clustering experiments were realized using anti-F3/contactin antibody-mediated cross-linking. In the absence of tunicamycin, pnd-GFP and F3/contactin were colocalized in clusters on the cell surface of the N2a cells (Fig. 5, D–F). In contrast, in the presence of tunicamycin, F3/contactin clusters were observed on the cell surface, whereas pnd-GFP was retained in the ER compartment (Fig. 5, G–I). It must be noted that the internal pool of F3/contactin cannot be visualized with antibody 24, which only recognizes F3/contactin by immunofluorescence on live cells.

Next, we analyzed the tunicamycin effect on the N-glycan content of caspr/paranodin and F3/contactin in transfected N2a cell extracts using Western blotting. As shown in Fig. 5J (lanes 5 and 6), tunicamycin treatment prevented N-glycosylation of F3/contactin. In the absence of tunicamycin, a doublet of 135–142-kDa glycosylated F3/contactin was detected, whereas a
single band of 130 kDa corresponding to the protein core was present after tunicamycin treatment. Unexpectedly, caspr/paranodin detected after tunicamycin treatment was still highly glycosylated (Fig. 5J, lanes 2 and 4). The deglycosylated protein core of F3/contactin expressed after tunicamycin treatment interacted with caspr/paranodin as demonstrated in co-
immunoprecipitation experiments and immunoblot analysis (Fig. 5F, lane 7).

Because we did not detect any deglycosylated form of caspr/paranodin at steady state after 24 h of tunicamycin treatment, we realized pulse-chase experiments to analyze the effect of tunicamycin on caspr/paranodin half-life. CHO cells stably co-transfected with F3/contactin and caspr/paranodin were treated for 2 h with tunicamycin, and radiolabeled with [35S]Met + Cys. Immunoprecipitation with anti-paranodin antiserum after the pulse showed that tunicamycin administration resulted in the biosynthesis of an unglycosylated form of caspr/paranodin at 170 kDa (Fig. 5K). A 135-kDa form of F3/contactin was detected in the immune precipitate in the absence of tunicamycin, whereas the deglycosylated form of 130 kDa was detected after tunicamycin treatment (Fig. 5K). Pulse-chases were performed during 2, 6, and 16 h to analyze the degradation rate of both proteins in complex (Fig. 5K). In the absence of tunicamycin, the half-life of caspr/paranodin was estimated at 4.5 h and tunicamycin strongly enhanced the degradation rate of the unglycosylated protein with a half-life reduced to 1 h (Fig. 5L). Under control conditions, co-immunoprecipitated F3/contactin appeared to be rapidly degraded, whereas in the presence of tunicamycin, the amount of deglycosylated F3/contactin coimmunoprecipitated with deglycosylated caspr/paranodin was enhanced (Fig. 5, K and L). These data demonstrate that the unglycosylated protein cores of caspr/paranodin and F3/contactin are interacting and that the sugar moieties reduce the binding activity between the two glycoproteins.

Thus, following long term treatment (24 h) with tunicamycin, a residual pool of glycosylated caspr/paranodin is detected in the ER (Fig. 5G) and the newly synthesized unglycosylated caspr/paranodin is rapidly degraded. However, in these conditions, a pool of F3/contactin is normally targeted to the cell surface independently of its N-glycan content (Fig. 5H). Our data indicate that N-glycosylation of caspr/paranodin induces its stabilization in the ER and that N-glycosylation of F3/contactin may be required for the cell surface targeting of the complex. One possibility could be that such export might be regulated via the calnexin/calreticulin cycle, which is blocked by tunicamycin (32).

The Lectin-Chaperone Calnexin Is Implicated in the Quality Control of Caspr/Paranodin—Retention-based, chaperone-mediated quality control in the ER has been well characterized in the case of glycoproteins. The activity of calnexin and calreticulin chaperones is based on a cycle of deglucosylation and reglucosylation that ensures a correct folding and assembly of glycoproteins. To analyze whether these chaperones are implicated in the folding of caspr/paranodin and its exit from the ER, we used castanospermine, which is known to inhibit the trimming of the three glucose from the core oligosaccharide and the subsequent association with calnexin or calreticulin. N2a cells were co-transfected with F3/contactin and caspr/paranodin and treated for 24 h with castanospermine. Castanospermine administration inhibited the cell surface expression of caspr/paranodin (Fig. 6B).

We hypothesized that F3/contactin binding may induce a transport-permissive conformation of caspr/paranodin, and interfere with the lectin-chaperone cycle. Using co-immunoprecipitation experiments, we showed that caspr/paranodin was associated with calnexin when stably transfected in CHO cells or transiently transfected in N2a cells (Fig. 6C, lanes 2 and 4, asterisks). In contrast, calnexin was not detected in the anti-paranodin immune precipitate from N2a cells co-transfected with F3/contactin and caspr/paranodin (Fig. 6C, lane 6). As a control for the specificity of calnexin interaction, N2a cells were transfected with caspr2, which is highly related to caspr/paranodin but is expressed on the cell surface independently of F3/contactin (see below Fig. 6A). As shown in Fig. 6C, lane 8, calnexin is not recovered in the anti-caspr2 immune precipitate.

Caspr/Paranodin and F3/Contactin Are Endo H-sensitive When Associated in Complex—Because N-glycosylation is implicated in the cell surface co-targeting of caspr/paranodin and F3/contactin, we have further analyzed the N-glycan content of the hetero-complex. The sensitivity to two glycosidases, PNGase F and Endo H, was assessed. PNGase F is an enzyme that removes specifically all N-linked glycans; Endo H only cleaves high mannose ER-type glycans but not complex glycans processed in the Golgi apparatus. PNGase F deglycosylation of the 180-kDa band of caspr/paranodin resulted in a −170-kDa band (Fig. 7A). Caspr/paranodin, which contains 15 putative N-gly-
cosylation sites, is highly N-glycosylated in N2a- or CHO-transfected cells. As previously described in Gennarini et al. (26), F3/contactin expressed in N2a or CHO-transfected cells is resolved as two bands of 142 and 135 kDa on SDS-PAGE, which correspond to two glycosylation variants. After deglycosylation with PNGase F, F3/contactin migrated as a single species with lower molecular mass (Fig. 7A). The lower band was Endo H-sensitive, whereas the upper band was Endo H-resistant, whereas the lower form of 135 kDa is Endo H-sensitive (lane 6). B, caspr/paranodin and F3/contactin are targeted to the cell surface with Endo H-sensitive N-glycans. Cell surface biotinylation was realized on double transfected CHO cells. After cell lysis, anti-paranodin immune precipitates were untreated (lanes 1 and 3) or incubated with Endo H (lanes 2 and 4). Immunoblots were revealed with anti-paranodin antiserum (lanes 1 and 2) or peroxidase-conjugated streptavidin (lanes 3 and 4). The total amount and the fraction of caspr/paranodin expressed at the cell surface are sensitive to Endo H digestion (arrowheads). Biotinylated F3/contactin co-immunoprecipitated (Ip) with caspr/paranodin is detected at 135 kDa and is Endo H-sensitive (asterisks). C, Endo H sensitivity of caspr/paranodin and F3/contactin in the lipid rafts from double transfected CHO cells. Triton X-100 cell lysates were subjected to an equilibrium centrifugation on sucrose gradient. Low density fractions containing rafts were untreated (lanes 1 and 3) or incubated with Endo H (lanes 2 and 4). Caspr/paranodin is Endo H-sensitive in the lipid rafts (lane 2). The two glycoforms of F3/contactin, bearing either Endo H-resistant or -sensitive carbohydrates are detected in the lipid raft fraction (lane 4). D, Endo H sensitivity of caspr/paranodin and F3/contactin in rat extracts. Microsomes extracted with Triton X-100, deoxycholate, and SDS were untreated (lanes 1 and 3) or incubated with Endo H (lanes 2 and 4). Caspr/paranodin is completely deglycosylated by Endo H treatment (lane 2). F3/contactin is expressed in rat brain microsomes as a single band of 135 kDa (lane 3) that is resolved as two bands following Endo H treatment (lane 4) indicating that both Endo H-resistant and Endo H-sensitive forms of F3/contactin are expressed in the rat brain. Experiments were made in triplicate. Wb, Western blot.

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Fig. 7. The glycocomplex of caspr/paranodin and F3/contactin is Endo H-sensitive. A, deglycosylation profiles of caspr/paranodin and F3/contactin in double transfected N2a cells. Nonidet P-40 lysates were untreated (lanes 1 and 4), or incubated with PNGase F (F) (lanes 2 and 5) or Endo H (H) (lanes 3 and 6). Immunoblotting with anti-paranodin antiserum (lanes 1-3) indicates caspr/paranodin is N-glycosylated (lane 2) and contains only Endo H-sensitive glycans (lane 3). Immunoblotting with anti-F3/contactin shows that the higher form of 142 kDa is Endo H-resistant, whereas the lower form of 135 kDa is Endo H-sensitive (lane 6). B, caspr/paranodin and F3/contactin are targeted to the cell surface with Endo H-sensitive N-glycans. Cell surface biotinylation was realized on double transfected CHO cells. After cell lysis, anti-paranodin immune precipitates were untreated (lanes 1 and 3) or incubated with Endo H (lanes 2 and 4). Immunoblots were revealed with anti-paranodin antiserum (lanes 1 and 2) or peroxidase-conjugated streptavidin (lanes 3 and 4). The total amount and the fraction of caspr/paranodin expressed at the cell surface are sensitive to Endo H digestion (arrowheads). Biotinylated F3/contactin co-immunoprecipitated (Ip) with caspr/paranodin is detected at 135 kDa and is Endo H-sensitive (asterisks). C, Endo H sensitivity of caspr/paranodin and F3/contactin in the lipid rafts from double transfected CHO cells. Triton X-100 cell lysates were subjected to an equilibrium centrifugation on sucrose gradient. Low density fractions containing rafts were untreated (lanes 1 and 3) or incubated with Endo H (lanes 2 and 4). Caspr/paranodin is Endo H-sensitive in the lipid rafts (lane 2). The two glycoforms of F3/contactin, bearing either Endo H-resistant or -sensitive carbohydrates are detected in the lipid raft fraction (lane 4). D, Endo H sensitivity of caspr/paranodin and F3/contactin in rat extracts. Microsomes extracted with Triton X-100, deoxycholate, and SDS were untreated (lanes 1 and 3) or incubated with Endo H (lanes 2 and 4). Caspr/paranodin is completely deglycosylated by Endo H treatment (lane 2). F3/contactin is expressed in rat brain microsomes as a single band of 135 kDa (lane 3) that is resolved as two bands following Endo H treatment (lane 4) indicating that both Endo H-resistant and Endo H-sensitive forms of F3/contactin are expressed in the rat brain. Experiments were made in triplicate. Wb, Western blot.

A commonly used method to inhibit protein transport through the Golgi apparatus is treatment of cells with brefeldin A, which interferes with the recruitment of the ADP-ribosylation factor-1 GTPase to coat protein complex I-coated membranes and prevents ER to Golgi transport (24). Treatment of N2a cells with brefeldin A effectively inhibited cell surface expression of caspr2, a transmembrane glycophorin highly related to caspr/paranodin (Fig. 8, A and B). However, brefeldin A treatment for 24 h did not inhibit the cell surface expression of either F3/contactin or caspr/paranodin (Fig. 8, C–F). It should be noted that using the same schedule of administration, both tunicamycin and castanospermine were effective to abolish the cell surface expression of caspr/paranodin. These results indicate that the complex of caspr/paranodin and F3/contactin is addressed to the cell surface via a Golgi-independent pathway.
transfection, cells were treated with 2.5 μg/ml brefeldin A for an additional period of 24 h (B, D, and F). After fixation and permeabilization with methanol, cells were immunostained for caspr2 (A and B) or caspr/paranodin (C and D). F3/contactin immunostaining was performed on live cells (E and F). Caspr2 is retained in an intracellular compartment of cells treated with brefeldin A (B). In contrast, both caspr/paranodin (D) and F3/contactin (F) are expressed at the surface of cells treated with brefeldin A.

Caspri/Paranodin Is Recruited into the Lipid Rafts with Endo H-sensitive Carbohydrates—We have previously demonstrated that the glycosylphosphatidylinositol anchor of F3/contactin is critically involved in the surface transport of caspr/paranodin via the lipid rafts (19). Spingolipid-cholesterol rafts form early in the ER and are part of the machinery ensuring correct intracellular trafficking of proteins (33, 34).

To address the nature of carbohydrates born by caspr/paranodin and F3/contactin in the lipid rafts, stably double transfected CHO cells were extracted with Triton X-100 and subjected to an equilibrium sucrose gradient centrifugation. Low density fractions corresponding to the lipid rafts were collected and incubated with Endo H. The two glycoylation variants of F3/contactin were detected in the lipid raft fraction; the lower form of F3/contactin was Endo H-sensitive, whereas the higher form was resistant (Fig. 7C). Caspr/paranodin, which was recruited into the lipid raft fraction through its binding with F3/contactin, was sensitive to Endo H (Fig. 7C). Therefore both forms of F3/contactin associate with raft domains even though they are sorted in distinct routes to the cell surface.

Caspri/Paranodin Is Endo H-sensitive in Brain Extracts—Caspri/paranodin is highly expressed in axonal paranodal junctions in the central nervous system of adult rats (12, 35). We investigated whether caspr/paranodin could be sensitive to Endo H in the brain, as observed in transfected CHO and N2a neuroblastoma cells. Particulate detergent extracts of adult rat brain were incubated with Endo H. F3/contactin from brain extracts migrated at 135 kDa, the same molecular mass that the lower form expressed in CHO cells, as described by Gennarini et al. (26). Endo H treatment resulted in two bands of 135 and 130 kDa, indicating that two pools of F3/contactin are present in the brain, one with Endo H-sensitive, the other with Endo H-resistant carbohydrates (Fig. 7D). In contrast, the total fraction of caspr/paranodin from brain extract was deglycosylated with Endo H (Fig. 7D).

Thus, the total pool of caspr/paranodin and a fraction of F3/contactin are expressed in the rat brain with Endo H-sensitive N-glycans. This result provides strong evidence that the complex of caspr/paranodin and F3/contactin may be addressed to the paranodal junctions via a non-conventional route, independently of the Golgi compartment.

DISCUSSION

We have demonstrated that the complex formed by caspr/paranodin and F3/contactin is delivered to the cell surface bearing high mannose N-glycans and in a brefeldin A-insensitive manner. These observations indicate that these glycoproteins may be addressed to paranodes via an unconventional pathway independent of the Golgi apparatus. Binding of F3/contactin induces an ER export permissive conformation of caspr/paranodin and its release from the lectin-chaperone calnexin. Our study suggests that the ER compartment is the site for control of caspr/paranodin and F3/contactin heterodimer assembly and for its sorting to a specific destination at the neuronal cell surface.

ER exit serves as an important checkpoint both in coordinating the assembly and stoichiometry of multisubunit protein complexes and in defining the amount of complexes expressed at the cell surface. For example, association with ER resident chaperones has been reported to control dimerization of immunoglobulin heavy and light chains, or assembly of procollagen chains (36, 37). The chaperone BiP binds specifically the constant region of the heavy IgG chain and deletion of this domain leads to loss of BiP binding and to secretion of the unassembled and otherwise transport-incompetent heavy chain. In the case of caspr/paranodin, the different deletions encompassing the whole extracellular region have not allowed the identification of any domain implicated in ER retention, or F3/contactin binding. Therefore, binding of F3/contactin may depend on the tridimensional structure of caspr/paranodin. In the absence of F3/contactin, caspr/paranodin is attached to the lectin-chaperone calnexin that confers ER residency. The cell surface delivery of caspr/paranodin is blocked by castanospermine administration as another indication of the role of the calnexin/calreticulin cycle in the quality control of this glycoprotein. We showed that N-glycosylated caspr/paranodin is stored as an intracellular pool and not addressed to lysosomes for degradation. Interaction with F3/contactin either induces a conformational change of caspr/paranodin or competes for calnexin binding, leading to ER exit of the heterodimer.

We provide evidence that two pools of F3/contactin are sorted in the ER toward two distinct routes. The high molecular weight F3/contactin is processed with Endo H-resistant carbohydrates and traffics to the cell surface via a Golgi-dependent pathway. We show that the fraction of F3/contactin associated with caspr/paranodin is diverted from the classical exocytic pathway. As shown by cell surface biotinylation, the low molecular weight F3/contactin forms a 1:1 association with caspr/paranodin. The complex is sorted to the cell surface with Endo H-sensitive carbohydrates and in a brefeldin A-insensitive manner, as a strong indication for a Golgi-independent pathway. In addition, this pathway can be blocked by tunicamycin treatment emphasizing the role of N-glycans in intracellular trafficking of caspr/paranodin. In contrast, cell surface delivery of F3/contactin via the Golgi-dependent pathway is insensitive to tunicamycin. It must be noted that both glycoforms of F3/contactin associate with raft domains even though they are sorted in distinct routes to the cell surface, indicating that raft association does not preclude for a specific pathway. In polar-
ized epithelial cell lines, glycosylphosphatidylinositol-anchored proteins are raft-associated and mainly apically delivered. However, some data indicate that, although the glycolipid anchor confers raft association, it is not sufficient to mediate apical sorting, whereas N-glycans can act as apical determinants of glycosylphosphatidylinositol-anchored proteins (38).

A direct transport from the ER to the cell surface that bypasses the Golgi has been recently reported for several molecules, including the transmembrane CD45 receptor (24), the palmitoylated raft component flotillin-1/reggie-2 (25), and the farnesylated K-Ras (39). The receptor protein-tyrosine phosphatase CD45 is expressed at the T cell surface as multiple isoforms and glycoforms. One glycoform is extremely rapidly expressed at the cell surface with Endo H-sensitive carbohydrates and in a brefeldin A-insensitive manner, whereas another form traffics through the conventional Golgi pathway (24).

Cell surface targeting of membrane components that bypass the Golgi apparatus may imply either a direct fusion between the peripheral components of the ER and the plasma membrane or involve a vesicle-mediated mechanism. A direct fusion mechanism would explain our observations that the cell surface expression of caspr/paranodin depends on the cell type. In neuroblastoma N2a cells, a close juxtaposition of ER elements with the plasma membrane would favor the whole surface targeting of the complex, whereas in double transfected CHO cells, which display less densely packed organelles, partial ER retention is observed. Apart from their role at the paranodal junctions, caspr/paranodin and F3/contactin are found at synaptic sites and may be implicated in a selective form of hippocampal synaptic plasticity (40). F3/contactin is essential for the membrane and synaptic targeting of caspr/paranodin in the hippocampus (40). In this context, the regulated exit of assembled complex from dendritic ER may provide a way to regulate the local targeting of CAMs at synaptic sites. The spine apparatus is continuous with smooth ER (41), offering the possibility of a direct fusion with the synaptic membrane. Such divergent routes for the two glycoforms of F3/contactin would provide a mechanism for selective renewal of the nodal and paranodal membrane components. As reported by Rios et al. (42), the two glycosylation variants of F3/contactin are distinctly expressed in the nodal domains. The high molecular weight form of F3/contactin would be targeted to the nodal gap via a Golgi-dependent pathway, whereas the low molecular weight form would traffic to paranodes via a non-conventional route. F3/contactin, which is expressed in the nodal gap in the central nervous system (4), may functionally interact with the sodium channels (42). In vitro studies indicate that F3/contactin binds the β1 accessory subunit and increases cell surface expression of Nav1.2 channels and sodium ionic current (43). F3/contactin is implicated in both the cell surface expression of sodium channel Nav1.2 and caspr/paranodin and thus it may play a pivotal role in the segregation of the different axonal subdomains. Multiple mechanisms may account for the formation of nodal domains including clustering of CAMs and channels at axo-glial contacts, and stabilization through linkage with cytoskeletal elements or regulated endocytosis. Our data indicate that differential trafficking toward the cell surface of nodal and paranodal glycoproteins may constitute an additional mechanism underlying the lateral segregation along the axon. Given the fence function of the septate-like junctions at paranodes that block lateral diffusion, such selective pathways would be especially critical for renewal of the distinct axonal subdomains at the nodes of Ranvier.

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REFERENCES


The Paranodal Complex of F3/Contactin and Caspr/Paranodin Traffics to the Cell Surface via a Non-conventional Pathway
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