PGY Repeats and N-Glycans Govern the Trafficking of Paranodin and Its Selective Association with Contactin and Neurofascin-155[□]

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Formation of nodes of Ranvier requires contact of axons with myelinating glial cells, generating specialized axo-glial subdomains. Caspr/paranodin is required for the formation of septate-like junctions at paranodes, whereas the related caspr2 is essential for the organization of juxtaparanodes. The molecular mechanisms underlying the segregation of these related glycoproteins within distinct complexes are poorly understood. Exit of paranodin from the endoplasmic reticulum (ER) is mediated by its interaction with F3/contactin. Using domain swapping with caspr2, we mapped a motif with Pro-Gly-Tyr repeats (PGY) in the ectodomain of paranodin responsible for its ER retention. Deletion of PGY allows cell surface delivery of paranodin bypassing the calnexin-calreticulin quality control. Conversely, insertion of PGY in caspr2 or NrCAM blocks these proteins in the ER. PGY is a novel type of processing signal that compels chaperoning of paranodin by contactin. Contactin associated with paranodin is expressed at the cell surface with high-mannose N-glycans. Using mutant CHO lines altered in the processing of N-linked carbohydrates, we show that the high-mannose glycoform of contactin strongly binds neurofascin-155, its glial partner at paranodes. Thus, the unconventional processing of paranodin and contactin may determine the selective association of axo-glial complexes at paranodes.

INTRODUCTION

Generation and rapid propagation of action potentials in myelinated fibers require a high density of voltage-gated sodium channels at initial segment and nodes of Ranvier. The organization of the nodes of Ranvier is achieved through multiple contacts between the axolemma and myelinating glial cells, generating specialized membrane subdomains with distinct composition in ion channels and cell adhesion molecules (Scherer and Arroyo, 2002; Poliak and Peles, 2003). The nodal gap is flanked by paranodes, characterized by septate-like junctions that seal the axonal membrane to the spiral of glial endfeet. The paranodal junctions act as fences to separate the nodal region from the juxtaparanodes enriched in potassium channels. The axonal glycoproteins caspr (contactin-associated protein)/paranodin and F3/contactin are key elements of the paranodal junctions (Einheber *et al.*, 1997; Rios *et al.*, 2000; Girault and Peles, 2002; Salzer, 2003) and interact in trans with the glial 155-kDa isoform of neurofascin (NF155; Charles *et al.*, 2002).

Genetic analyses indicate that deficiency in either paranodin, contactin, or NF155 results in the disorganization of the paranodal loops and a reduction in nerve conduction velocity (Bhat *et al.*, 2001; Boyle *et al.*, 2001; Sherman *et al.*, 2005).

Caspr2, which exhibits a 42% sequence identity with paranodin, is enriched at juxta-paranodes in association with a cell adhesion molecule of the Ig superfamily (IgCAM) closely related to contactin, TAG-1, and both molecules are required for the clustering of potassium channels at juxta-paranodes (Poliak *et al.*, 2003; Traka *et al.*, 2003). The strikingly distinct location of highly related paranodin/contactin and caspr2/TAG-1 complexes indicates the existence of very efficient mechanisms of segregation along the axonal membrane. One possibility is that these cell adhesion molecules are uniformly targeted to the axonal membrane and become clustered at their respective final location through axo-glial interactions and cytoskeletal linkers. Alternatively, preformed complexes of paranodal or juxta-paranodal components may be sorted to their specific subdomains.

The cell surface delivery of paranodin and contactin is a tightly controlled process. Paranodin and contactin are interdependent for their distribution at the paranodes, because the knockout mutation of contactin in mice prevents intracellular transport and surface expression of paranodin, which becomes confined to neuronal cell bodies (Boyle *et al.*, 2001). In the absence of contactin, paranodin is blocked in the endoplasmic reticulum (ER; Faivre-Sarrailh *et al.*, 2000). Association with the GPI-anchored contactin releases paranodin from the lectin-chaperone calnexin, allowing the com-

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plex to exit from the ER (Bonnon *et al.*, 2003). The ER quality control checkpoints provide a stringent process to eliminate misfolded proteins and prevent premature export of unassembled subunits (Ellgaard and Helenius, 2003). Proteins are subjected to a primary quality control based on the recognition of conformational features such as exposure of hydrophobic regions, unpaired cysteine residues, or the tendency to aggregate. More specific mechanisms control ER retention or retrieval from the Golgi and ER exit of particular classes of proteins. Cytoplasmic retention signals such as dibasic motifs are masked by correct oligomeric assembly to determine the surface expression of functional receptors and ion channels (Zerangue *et al.*, 1999; Bichet *et al.*, 2000; Standley *et al.*, 2000).

Here, using a combination of deletions and domain swapping between paranodin and caspr2, we identify the extracellular region of paranodin responsible for its ER retention. This region was termed PGY because it includes a 10-fold imperfect repetition of Pro-Gly-Tyr. Our study indicates that PGY acts as a conformation-based retention signal that compels chaperoning by contactin and the calnexin/calreticulin cycle before export of the paranodal glycoprotein complex. Importantly, contactin associated with paranodin is processed with ER-type high mannose N-glycans, generating a glycoform that selectively binds NF155.

MATERIALS AND METHODS

Antibodies

Rabbit antiserum SL51 reacted with epitopes in the intracellular region of paranodin (Menegoz *et al.*, 1997). The anti-caspr2 antibody was generated by immunizing rabbits with the intracellular region (residues 1284–1331) fused to GST (Traka *et al.*, 2003). We used a rabbit antiserum directed against amino acids 37–50 of F3/contactin for immunoblotting (Rigato *et al.*, 2002) and antiserum 24 for immunostaining (Durbec *et al.*, 1994). Mouse anti-GFP and rat anti-HA mAbs were from Roche (Meylan, France), rabbit anti-calnexin antiserum from Transduction Laboratories (BD Biosciences, San Jose, CA), mouse anti-BiP (KDEL) mAb from StressGen Biotechnologies (Victoria, BC, Canada) and mouse anti-58K mAb from Sigma (St-Quentin Fallavier, France). Alexa-488–, -568–, and -647–conjugated secondary antibodies were from Molecular Probes (Eugene, OR). Peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA).

Cloning Strategies

The DNA construct pRc-CMV/F3 encoding the full-length sequence of F3/contactin (Durbec *et al.*, 1994) and pBK-CMV-pnd encoding the full-length sequence of paranodin (Menegoz *et al.*, 1997) were described previously. The DNA coding for the HA-tagged Arf1(Q71L) inserted into pSR2 was a gift from Dr. P. Chavrier (Prigent *et al.*, 2003). The full-length sequence of caspr2 was inserted at SalI/NotI sites into pBlueScriptII SK and subcloned in pCDNA3 at the KpnI/NotI sites (Bonnon *et al.*, 2003). The caspr2N-pnd chimera was generated by PCR amplification with the Pfu polymerase (Roche) of the discoidin and first laminin G (LNG-1) domains of caspr2 (aa 1–275) inserted into the SnaBI sites of pBK-CMV-pnd. The pnd-caspr2C chimera was generated by PCR amplification of the LNG-4, transmembrane, and intracellular domains of caspr2 (aa 1029–1331) inserted into the PmlI/XbaI sites of pBK-CMV-pnd. The pndΔPGY1 construct was obtained by PCR amplification of the LNG-2 and EGF-2 domains of paranodin (aa 783–1030) inserted at the BstEII site into pBK-CMV-pndΔ4, previously described in Bonnon *et al.* (2003). The pndΔPGY2 construct was generated by PCR amplification of the LNG-4 and intracellular domains of paranodin (aa 1082–1381) inserted into the PmlI/XbaI sites of the pnd-caspr2C chimera. The pndΔ985–1030 construct was generated by PCR amplification of the PGY, LNG-4, and intracellular domains of paranodin (aa 1031–1381) inserted into the PmlI/XbaI sites of pBK-CMV-pnd. The caspr2-PGY construct was generated by PCR amplification of part of the EGF-2 and the PGY repeats domains of paranodin (aa 992–1116) inserted into the PmlI site of pBS/caspr2 and subcloned into the EcoNI-NotI sites of pCDNA3/caspr2. The NrCAM-PGY construct was generated by PCR amplification of the PGY repeats domains of paranodin (aa 1014–1083) fused to the fourth FNIII-C-terminal region of NrCAM (aa 944–1215) inserted at XhoI/BamHI sites of the NrCAMΔFnΔcyt construct (Falk *et al.*, 2004). The contactin-GFP construct was obtained by subcloning the HindIII/EcoRI sequence from pRc-CMV/F3 in pEGFP-N2 (Clontech, Mountain View, CA), resulting in the in frame fusion of GFP at the C-terminus of the complete coding sequence of contactin (aa 1–1020). The

PCR-amplified products were verified by sequencing (Genome Express, Meylan, France).

Cell Culture

COS-7, CHO, and neuroblastoma N2a cells grown in DMEM containing 10% FCS were transiently transfected using jet PEI (Polyplus transfection, Illkirsch, France). Transfected N2a cells were cultured for 24 h in OptiMEM (Invitrogen, Cergy Pontoise, France) before treatments with 2 μg/ml tunicamycin (Sigma) or 1 mM castanospermine (Sigma) during an additional period of 18 h. The parental (pro5[−]) and N-glycosylation mutant CHO cell lines (Lec1, Lec10, and Lec23; Stanley and Ioffe, 1995) were kindly provided by Dr. E. Fenouillet (Institut Jean Roche, Marseille, France). The NF155-Fc (Charles *et al.*, 2002) and NrCAM-Fc (Faivre-Sarraillh *et al.*, 1999) were produced in the supernatant of transfected COS-7 cells and used as previously described for binding or ligand-affinity purification experiments. Immunoprecipitation, endoglycosidase treatments, cell surface biotinylation were performed as previously described (Bonnon *et al.*, 2003).

Immunofluorescence and Confocal Microscopy

N2a cells plated on glass coverslips were transfected with the different constructs. The distribution of caspr2, paranodin, caspr2N-pnd, pnd-caspr2C, pndΔPGY1, pndΔPGY2, pndΔ985–1030, and caspr2-PGY was analyzed on N2a-transfected cells using anti-paranodin or anti-caspr2 antibodies. For double-staining with anti-BiP or anti-HA mAb, cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 (TX-100) for 10 min. For double-staining with anti-58K mAb, cells were fixed with methanol for 10 min at −20°C. Immunofluorescence staining was performed using SL51 (1:2000), anti-caspr2 (1:2000), anti-BiP (1:200), anti-HA (1:100), or anti-58K (1:100) antibodies diluted in PBS containing 3% bovine serum albumin (BSA). The cells were rinsed with PBS and incubated with secondary antibodies diluted in PBS containing 3% BSA for 30 min. After washing in PBS, cells were mounted in Mowiol (Calbiochem, La Jolla, CA).

Confocal image acquisition was performed on a Leica (Wetzlar, Germany) TCS SP2 laser scanning microscope equipped with 63×/1.32 na oil immersion objective. Images of GFP or Alexa-488–, -568–, and -647–conjugated antibodies stained cells were obtained using, respectively, the 488-nm band of an Argon laser and the 543- and 633-nm bands of an He-Ne laser for excitation. Spectral detection and emitted fluorescence was set as follows: 500–535 nm for Alexa-488 or GFP, 550–620 nm for Alexa-568 and 650–750 nm for Alexa-647. Fluorescence images were collected automatically as frame-by-frame sequential series, each image being produced from an average of six frame scans. Pixel size was set to 163 nm by adjusting the electronic zoom at 2.8×. Three-dimensional (3D) stacks of confocal images were acquired using a 1-μm step, and representative images were selected.

For the quantitative analysis of NF155-Fc binding, fluorescence images were collected in randomly selected area with identical parameters of acquisition using a Nikon (Champigny sur Marne, France) E800 microscope equipped with epifluorescence and an Hamamatsu Photonics (Massy, France) ORCA-ER camera. The mean of fluorescence was measured on individual cells using the LUCIA (Nikon) image analysis software.

NMR Spectroscopy of the PGY-rich Sequence

A peptide of 22-mer corresponding to half of sequence of the PGY repeats (aa 1034–1055, PGYEPGYIPGYDTPGYVPGYHG) was purchased from EZBiolab (Westfield, IN). 2D-NMR spectra of this PGY repeat peptide (2 mM in 500 μl of H₂O/D₂O, 90/10, vol/vol) were recorded on a Bruker DRX500 spectrometer (Rheinstetten, Germany) equipped with an HCN probe and self-shielded triple axis gradients. A TOCSY (clean total correlation spectroscopy) with a spin-lock time of 80 ms and a spin-locking field strength of 8 kHz, a NOESY (nuclear Overhauser effect spectroscopy) with a mixing time of 150 ms and a ROESY (rotational nuclear Overhauser effect spectroscopy) with a mixing time of 80 ms were acquired. Water suppression was obtained with a water-gate 3–9–19 pulse train using a gradient at the magic angle obtained by applying simultaneous x-, y-, and z-gradients before detection.

Online Supplementary Material

Supplementary Table S1 shows the quantitative analysis of the kinetics of cell membrane expression of paranodin/caspr2 chimeras and mutant forms after transfection in N2a cells. Supplementary Figure S1 shows that the transmembrane domain of paranodin is not implicated in ER retention. Supplementary Figure S2 shows that contactin-GFP interacted with paranodin and induced its expression at the cell membrane. The structure prediction of the PGY region is shown in Supplementary Figure S3.

RESULTS

The C-terminal Region of Paranodin Ectodomain Is Implicated in ER Retention

When expressed alone in neuroblastoma N2a cells, paranodin is blocked in the ER (Faivre-Sarraillh *et al.*, 2000). The

Figure 1. Schematic representation of paranodin/caspr2 chimeras and mutant constructs. (A) Modular organization of paranodin (pnd), caspr2, and mutated constructs. Pnd Δ PGY1 is deleted of PGY and the beginning of the fourth laminin G (LNG-4) domain (aa 1031–1097). Pnd Δ PGY2 is deleted of part of the second EGF (EGF-2) domain, adjacent interdomain, and PGY motif (aa 985–1081). Pnd Δ 985–1030 is deleted of part of EGF-2 and adjacent interdomain (aa 985–1030). In the caspr2N-pnd chimera, the N-terminal part of paranodin including the discoidin and LNG-1 domains (aa 1–275) is replaced by the corresponding domains of caspr2. The pnd-caspr2C chimera is composed of the N-terminal region of paranodin up to half of the EGF-2 domain (aa 1–984) fused with the C-terminal domain of caspr2 from the LNG-4 domain (aa 1029–1331). The caspr2-PGY construct contains an insertion of a paranodin sequence including half of the EGF-2 domain, PGY, and the beginning of the LNG-4 domain (aa 992–1116) into caspr2. The NrCAM-PGY construct contains the signal peptide and the 6 Ig domains of NrCAM, the GFP and the PGY motif (aa 1014–1083) fused with the C-terminal region of NrCAM from the fourth fibronectin type III up to the transmembrane domain. (B) Putative N-glycosylation sites are indicated along the sequences of paranodin (15 sites) and caspr2 (10 sites). Note that only two of these sites (●) are conserved in the two proteins.

retention of paranodin in the ER is not due to its cytoplasmic tail (Bonnon *et al.*, 2003). Although paranodin transmembrane region contains a sequence (VLFYxxN) similar to an ER retention motif (PLFYxxN) described in the acetylcholine receptor subunits (Wang *et al.*, 2002), the transmembrane region was not involved in the ER retention of paranodin (Supplementary Figure S1). To examine precisely the role of the ectodomain of paranodin in its trafficking, we used domain swapping between paranodin and caspr2. Caspr2 exhibits high sequence homology with paranodin, but does not interact with contactin and is addressed to the cell surface via the classical Golgi-dependent pathway (Bonnon *et al.*, 2003). We generated two chimeras, caspr2N-pnd and pnd-caspr2C, in which the N- or C-terminal region of paranodin was replaced by the homologous region of caspr2 (Figure 1A). When transiently transfected in N2a cells, paranodin alone was retained in the ER and colocalized with the ER chaperone BiP (Figure 2, A and B). In contrast, paranodin cotransfected with contactin was expressed at the cell membrane (Figure 2, C and D). Caspr2 was essentially detected at the cell membrane (Figure 2, E and F). The caspr2N-pnd chimera was colocalized with BiP and restricted to the ER (Figure 2, G and H). In contrast, pnd-caspr2C was essentially expressed at the cell membrane in most cells (60%, $n = 118$), with only a small fraction of the protein detected in the ER (Figure 2, I and J). These data provide strong evidence that the region of the ectodomain of paranodin C-terminal to the EGF-2 domain is implicated in ER retention.

An Extracellular Region with Pro-Gly-Tyr Repeats Mediates the Retention of Paranodin in the ER

Comparison of the C-terminal sequences of the ectodomains of paranodin and caspr2 reveals a ~50-residue region with 10 imperfect repeats of the triplet Pro-Gly-Tyr followed by one or two other residues, here termed PGY. This region is absent in caspr2 (Figure 3A). To test for the possible role of

PGY in ER retention, we investigated the consequences of PGY deletion in paranodin. Pnd Δ PGY1 deleted of residues 1031–1097 (Figures 1A and 3A) was colocalized with the ER marker BiP and was not detected at the cell membrane in transfected N2a cells (Figure 3, B and C). We generated pnd Δ PGY2, a mutant with a larger deletion (lacking residues 985–1081), corresponding to that in pnd-caspr2C (Figure 1A), including part of the EGF-2 domain and the sequence upstream of PGY (Figure 3A). Pnd Δ PGY2 was strongly expressed at the cell membrane in a low percentage of transfected cells (21%, $n = 245$) 48 h after transfection (Figure 3, D and E). We generated pnd Δ 985–1030 to evaluate the possible role of the sequence upstream of PGY, which is deleted in the pnd Δ PGY2 construct (Figure 1A). Pnd Δ 985–1030 was distributed in the ER and was never detected at the cell membrane (Figure 3, F and G). Altogether these results demonstrate that ER retention of paranodin requires an extracellular signal that includes the PGY region.

Insertion of PGY in caspr2 and NrCAM Induces ER Retention

The above results indicated that the presence of PGY was necessary to retain paranodin in the ER. We tested whether this motif acted as an ER retention signal when introduced in caspr2, which normally traffics to the cell surface. We generated caspr2-PGY by insertion of a sequence containing PGY (aa 992–1116) into caspr2 at a position homologous to its location in paranodin (Figure 1A). Although a faint staining was detected at the cell membrane of some cells, caspr2-PGY was mostly retained in the ER and colocalized with BiP in the majority of transfected N2a cells (62%, $n = 105$), as shown in Figure 3, H and I. Next, we investigated whether PGY might act as an ER retention signal when introduced in a structurally unrelated molecule, NrCAM, a cell adhesion molecule of the Ig superfamily. PGY (residues 1014–1083) was inserted in the extracellular region of a GFP-tagged

Figure 2. Mapping the ER retention motif in the extracellular region of paranodin. N2a cells were transiently transfected with paranodin (pnd; A and B), paranodin and contactin (C and D), caspr2 (E and F), caspr2N-pnd (G and H), or pnd-caspr2C (I and J) and fixed 48 h after transfection. (A, C, E, G, and I). Cells fixed with methanol were immunostained using anti-paranodin or anti-caspr2 antiserum (green). (B, D, F, H, and J) Cells fixed with paraformaldehyde and permeabilized with TX-100 were double-immunostained using anti-paranodin or anti-caspr2 antiserum (red) and for the ER marker BiP (green). When expressed alone, paranodin is retained in the ER, colocalized with BiP (B). In contrast, when cotransfected with contactin, paranodin is enriched at the cell membrane (C and D). Caspr2 is detected at the cell membrane (E and F). Caspr2N-pnd is only detected in the ER, colocalized with BiP (H). Pnd-caspr2C is mainly expressed at the cell membrane but is also slightly detected in the ER (I and J). Images were obtained using a Leica confocal microscope with 63 $\times$ /1.3NP lens. A representative image has been selected from the z-stack using a 2 $\times$ zoom in A, C, E, G, and I (bar, 30 μ m) and 2.8 $\times$ zoom in B, D, F, H, and J (bar, 10 μ m).

NrCAM chimera (Falk *et al.*, 2004), between the globular domains of GFP and the fibronectin type III repeats, at a similar distance from the transmembrane domain as in paranodin (140 aa vs. 200 aa in paranodin; Figure 1A). The NrCAM-PGY construct was mostly retained in the ER of N2a transfected cells (86%, $n = 147$, Figure 3K), whereas the control construct was only detected at the cell membrane (Figure 3J). Therefore, adding PGY was sufficient to induce massive ER retention of caspr2 and NrCAM.

The role of PGY as an ER retention signal was also analyzed using a cell surface biotinylation assay in N2a cells transfected with paranodin, caspr2, or mutant constructs (Figure 4). Consistent with its intracellular localization, paranodin was slightly biotinylated (Figure 4, lane 1). Similarly, pnd Δ PGY1 and pnd Δ 985–1030, which were only detected in the ER, were slightly biotinylated (lanes 2 and 3). In contrast, biotinylated pnd-caspr2C chimera (lane 7) and pnd Δ PGY2 (lane 4) were detected at high levels in the immune precipitate, indicating that deletion of PGY allowed cell surface expression of the mutant protein. Caspr2, which is expressed at the cell surface, was highly biotinylated (Figure 4, lane 5), whereas only a low amount of biotinylated caspr2-PGY could be detected (Figure 4, lane 6). Thus, these biochemical data confirm the crucial role of PGY in the retention of paranodin in the ER.

Deletion of PGY Allows Bypass of the Quality Control by the Lectin Chaperones Calnexin and Calreticulin

Cell surface targeting of the paranodin and contactin complex requires its interaction with the lectin chaperones cal-

nexin and calreticulin (Bonnon *et al.*, 2003). Accordingly, the cell surface transport of paranodin is prevented by treatment with castanospermine (Bonnon *et al.*, 2003), which blocks the calnexin and calreticulin cycle by inhibiting glucosidases I and II (Spiro, 2004). After castanospermine treatment, the cell membrane expression of paranodin cotransfected with contactin was blocked (Figure 5B), whereas caspr2 was still expressed at the cell membrane (Figure 5E). In marked contrast with paranodin, pnd Δ PGY2 and pnd-caspr2C, which both lacked PGY, were delivered to the plasma membrane in the presence of castanospermine (Figure 5, H and K): pnd Δ PGY2 was expressed at the cell membrane of 26% of the cells ($n = 115$) in the absence of castanospermine and of 25% of the cells ($n = 114$) in its presence; pnd-caspr2C was expressed at the cell membrane of 67% of the cells ($n = 106$) in the absence of castanospermine and of 63% of the cells ($n = 124$), in its presence. Thus deletion of PGY allowed to bypass the quality control by calnexin and calreticulin.

Paranodin expressed alone strongly associates with calnexin, whereas its association with contactin allows dissociation of the chaperone (Bonnon *et al.*, 2003). Coimmunoprecipitation experiments were performed to analyze whether the PGY repeats mediate the interaction of paranodin with calnexin (Figure 5M). Calnexin was coimmunoprecipitated with paranodin (lane 2) and to a lesser extent with pnd Δ PGY2 (lane 4). Calnexin did not interact with caspr2 (lane 6) and interacted weakly with pnd-caspr2C (lane 8). These data indicate that the PGY repeats in paranodin do not directly mediate calnexin binding but may be a conformational signal, inducing ER retention of paranodin at the

Figure 3. A motif with Pro-Gly-Tyr repeats (PGY) is implicated in the ER retention of paranodin. (A) Sequence alignment of the C-terminal region of the ectodomain of the rat paranodin (ratpnd) and human caspr2 (hucaspr2). A sequence that contains 10 imperfect repeats of Pro-Gly-Tyr (pink) is present between the EGF-2 (gray) and LNG-4 (dark gray) domains of paranodin but not of caspr2. The region deleted in pndΔPGY1 is indicated with a red dashed line and the one deleted in pndΔPGY2 is indicated with a black dashed line. (B–I) Confocal analysis of N2a cells transfected with pndΔPGY1 (B and C), pndΔPGY2 (D and E), pndΔ985–1030 (F and G), or caspr2-PGY (H and I) and fixed 48 h after transfection. (B, D, F, and H) Cells fixed with methanol were immunostained using anti-paranodin or anti-caspr2 antiserum (green). (C, E, G, and I) Cells fixed with paraformaldehyde and permeabilized with TX-100 were double-immunostained using anti-paranodin or anti-caspr2 (red) and anti-BiP (green) antiserum. PndΔPGY1 is retained in the ER (B and C). A different deletion including a sequence upstream of PGY results in the cell membrane expression of pndΔPGY2 (D and E). Deletion of PGY is required for ER exit because in pndΔ985–1030, a shorter deletion restricted to the sequence upstream of PGY does not prevent ER retention (F and G). Reciprocally, insertion of the PGY repeats in caspr2, which normally traffics to the cell surface, induces the ER retention of the caspr2-PGY chimera (H and I). (J and K) N2a cells transfected with NrCAM-GFP and NrCAM-PGY. Insertion of PGY in the extracellular region of NrCAM-GFP results in ER retention of NrCAM-PGY (K), whereas the NrCAM-GFP control construct is only detected at the cell membrane (J). Cells were fixed with paraformaldehyde and confocal images of the GFP fluorescence were collected. Bar, 30 μ m in B, D, F, H, J, and K and 10 μ m in C, E, G, and I.

quality control checkpoint mediated by the calnexin/calreticulin cycle.

When inserted ectopically in caspr2 or NrCAM, the PGY repeats induced ER retention in most of the cells (Figure 3, H and K). Castanospermine treatment further increased ER retention of caspr2-PGY, which was expressed at the cell surface of 11% of the cells ($n = 123$) in the presence of castanospermine compared with 42% of the cells ($n = 92$) under control conditions. Thus, insertion of PGY in caspr2 induced for a part chaperoning through the calnexin/calreticulin cycle. However, calnexin was not detected in the immune precipitate of caspr2-PGY (Figure 5M, lane 10), likely because the interaction between caspr2-PGY and the lectin chaperone may be transient. In contrast, the cell surface delivery NrCAM-PGY was not modified by castanospermine treatment (not shown). Therefore, PGY by itself does not determine the selective association with calnexin/calreticulin lectin chaperones, which may de-

pend on the adjacent regions conserved in other paranodin family members.

Because proline-rich sequences are known to participate in multiple protein-protein interactions (Williamson, 1994), we examined by immunoprecipitation whether the PGY region was involved in contactin binding. Both pndΔPGY2 and pnd-caspr2C, which lack PGY, associated with the 135-kDa form of contactin (Figure 6A, lanes 3 and 5), indicating that PGY was not required for the association of paranodin with contactin. However, coexpression of contactin with pndΔPGY2 or pnd-caspr2C did not modify the cell surface expression of the mutated proteins in the absence or presence of castanospermine (not shown).

We then examined whether the presence of Pro-Gly-Tyr repeats favored an identifiable 3D structure in solution by analyzing the conformation of a 22-mer peptide corresponding to half of the PGY motif (aa 1034–1055) by NMR spectroscopy. Amide proton resonance frequencies were distrib-

Figure 4. Role of the PGY repeats analyzed using cell surface biotinylation. Cell surface biotinylation was carried out on N2a cells transfected with paranodin (pnd), pnd Δ PGY1, pnd Δ 985–1030, pnd Δ PGY2, caspr2, caspr2-PGY, or pnd-caspr2C as indicated. Paranodin, pnd Δ PGY1, pnd Δ 985–1030, and pnd Δ PGY2 were immunoprecipitated with an anti-paranodin antibody (lanes 1–4). Caspr2, caspr2-PGY, and pnd-caspr2C were immunoprecipitated with an anti-caspr2 antibody (lanes 5–7). Proteins were revealed with anti-paranodin or anti-caspr2 antiserum, or with peroxidase-conjugated streptavidin. Paranodin, pnd Δ PGY1, and pnd Δ 985–1030 are slightly biotinylated (lane 1–3). In contrast, pnd-caspr2C (lane 7) and pnd Δ PGY2 (lane 4) are strongly biotinylated in the immune precipitate, indicating that they are expressed at the plasma membrane. Biotinylated caspr2 is strongly detected using streptavidin (lane 5), whereas the biotinylated form is dramatically decreased for caspr2-PGY (lane 6), indicating that insertion of PGY inhibits the cell surface expression of the chimera. Ip, immunoprecipitation; Wb, Western blot.

uted in a range as narrow as 1 ppm (ranging from 7.5 to 8.5 ppm). The observed line width (4.5 Hz) was small, the efficiency of the scalar coupling observed in the TOCSY (all spin systems are complete) was high, and there was no correlation except for the intrareidue ones in both the NOESY and the ROESY experiments. These features clearly indicated a peptide with high internal mobility and devoid of any stable conformation, although we could not rule out that the full-length PGY motif takes a more organized structure. Therefore, we analyzed the entire PGY motif (aa 1027–1085) using the Rosetta de novo structure prediction method (Yarov-Yarovoy *et al.*, 2006). The program predicted a β -sheet-based globular structure, which was stable when studied by molecular dynamics (Supplementary Figure S3; see also Figure 9A). This structure was even more stable when the linker between the PGY-rich region and the EGF-2 domain was included in the model, as well as the adjacent EGF-2 and LNG-4 domains. It should be noted that the short PGY peptide (aa 1034–1055), which lacked structural organization identifiable by NMR, adopted coil conformations, escaping rapidly from its starting β -sheets. These results suggest that the PGY region has the capacity to adopt a stable globular structure.

N-Glycosylation Controls the Cell Membrane Delivery of Paranodin

N-glycosylation of membrane proteins is implicated in a series of events along the exocytosis pathway, including regulation of folding and trafficking (Helenius and Aebi,

Figure 5. Quality control by the calnexin/calreticulin cycle and role of N-glycosylation in the cell membrane delivery of paranodin. N2a cells were transfected with paranodin (pnd) and contactin (A–C), caspr2 (D–F), pnd Δ PGY2 (G–I), or pnd-caspr2C (J–L). Cells were treated 24 h after transfection with 1 mM castanospermine (B, E, H, and K) or 2 μ g/ml tunicamycin (C, F, I, and L) for an additional 18-h period. In double-transfected N2a cells, paranodin is retained in the ER in presence of castanospermine (B), which inhibits the calnexin/calreticulin cycle. Castanospermine treatment does not prevent cell membrane expression of caspr2 (E). Likewise, pnd Δ PGY2 (H) and pnd-caspr2C (K) expression at the cell membrane is insensitive to castanospermine. Tunicamycin treatment, which blocks the first step of N-glycosylation, results in ER retention of paranodin in double-transfected N2a cells (C) and has no effect on the cell membrane expression of caspr2 (F). Both pnd Δ PGY2 (I) and pnd-caspr2C (L) are blocked in the ER after tunicamycin treatment. Bar, 10 μ m. (M) COS-7 cells were transfected with paranodin, caspr2, or mutant constructs, as indicated. After cell lysis with NP-40, immunoprecipitation was carried out using anti-paranodin antiserum for paranodin (pnd) and pnd Δ PGY2 (lanes 1–4) or anti-caspr2 antiserum for caspr2, pnd-caspr2C, and caspr2-PGY (lanes 5–10). Wb, Western blot.

Figure 6. Interaction of paranodin/caspr2 chimeras with contactin and sensitivity to endoglycosidases. (A) Immunoprecipitation from NP-40 lysates of transfected COS-7 cells was carried out using anti-paranodin antiserum for paranodin and pndΔPGY2 (lanes 2 and 3) or anti-caspr2 antiserum for caspr2 and pnd-caspr2C (lanes 4 and 5). Proteins were detected in the lysates (L) and immunoprecipitates (ip) using anti-contactin antiserum. Contactin migrates as a doublet of 135 and 142 kDa in the lysate. Only the 135-kDa form, which is Endo H-sensitive, is detected in the immune precipitate of paranodin, pndΔPGY2, and pnd-caspr2C. As a control, contactin does not coimmunoprecipitate with caspr2. Wb, Western blot. (B) N-glycosylation profiles of paranodin, caspr2, and mutant constructs in transfected COS-7 cells. NP-40 lysates were untreated (–) or incubated with PNGase F (F) or Endo H (H), and immunoblotting was realized with anti-paranodin (lanes 1–3) or anti-caspr2 (lanes 4–12) antiserum. Decreased apparent molecular weight after PNGase F treatment indicates that proteins are N-glycosylated. Paranodin is Endo H-sensitive (lane 3), whereas caspr2 is Endo H-resistant (lane 6). In accordance with its ER distribution, caspr2-PGY is Endo H-sensitive (lane 9). Interestingly, the pnd-caspr2C chimera displays both Endo H-resistant and Endo H-sensitive forms (lane 12).

2004). Paranodin and caspr2 contain several putative sites of N-glycosylation, only two of which are conserved between the two molecules (Figure 1B). Cell surface delivery of paranodin depend on N-glycans, because tunicamycin, a specific inhibitor of N-glycosylation, induces ER retention of paranodin (Bonnon *et al.*, 2003). We examined whether this requirement for N-glycosylation was related to the presence of the PGY motif. Tunicamycin treatment blocked paranodin in the ER of N2a cells cotransfected with contactin (Figure 5C). In contrast, caspr2 was readily targeted to the cell membrane after tunicamycin treatment (Figure 5F). Tuni-

camycin completely prevented the cell membrane expression of pndΔPGY2 (Figure 5I) and pnd-caspr2C (Figure 5L) and induced their ER retention in 100% (n = 133) and 91% (n = 130) of transfected cells, respectively. These results show that N-glycosylation is required for the cell surface transport of paranodin, even in the absence of PGY.

The paranodin–contactin complex expressed at the cell surface contains mannose-rich ER-type N-glycans that are sensitive to endoglycosidase H (Endo H; Bonnon *et al.*, 2003). We examined the sensitivity of the various chimeras to Endo H, as compared with peptide N-Glycosidase F (PNGase F) that deglycosylates both ER and Golgi-type glycoproteins. Both PNGase F and Endo H deglycosylated paranodin, shifting its apparent molecular weight from 180 to 170 kDa (Figure 6B, lanes 2 and 3), whereas caspr2 was Endo H-resistant (Figure 6B, lane 6). Caspr2-PGY was sensitive to Endo H (Figure 6B, lane 9), in agreement with its retention in the ER. The pnd-caspr2C chimera, which was delivered to the cell surface, was resolved in two bands, a 170-kDa Endo H-sensitive and a 180-kDa Endo H-resistant bands (Figure 6B, lane 12), an indication that it was partly processed through the Golgi apparatus.

COPI-mediated Processing of Paranodin Associated with Contactin

We previously showed that paranodin expression at the cell membrane was insensitive to brefeldin A (Bonnon *et al.*, 2003), a drug that inhibits COPI-mediated transport by interfering with Arf1 GTPase (Lippincott-Schwartz *et al.*, 1989). This result suggested an unconventional routing of paranodin bypassing the Golgi apparatus. We further explored the possibility of a COPI-mediated transport of paranodin using cotransfection with the dominant negative Arf1-GTP(Q71L) (Prigent *et al.*, 2003). COPI regulates anterograde transport from the ER to the Golgi stack, transport within the Golgi stack, and retrograde transport of recycling components from pre-Golgi and Golgi membranes to the ER (Rabouille and Klumperman, 2005). The dominant negative Arf1(Q71L) is locked in the GTP-bound form that blocks COPI coat disassembly. Arf1(Q71L) strongly inhibited the cell membrane expression of paranodin cotransfected with contactin, which accumulated into Golgi structures 14 h after transfection (Figure 7, A and G). It must be noted that the ER-resident chaperone BiP, which contains a KDEL motif of retrieval from the Golgi to the ER, also accumulated in the Golgi apparatus of cells expressing Arf1(Q71L) (Figure 7B). Thus, the complex of paranodin and contactin may recycle through COPI vesicles from the Golgi to the ER, possibly in association with chaperones. In contrast, paranodin cotransfected with Arf1(Q71L) was not recruited into the Golgi in the absence of contactin, indicating that the ER retention of paranodin expressed alone did not depend on retrieval from the Golgi (Figure 7C).

Paranodin Does Not Accumulate in the Golgi after Temperature Blockade of Export

We analyzed the kinetics of transport to the cell membrane of contactin, paranodin, caspr2, and their mutated forms in transfected N2a cells (Supplementary Table S1). Because the anti-contactin antiserum 24 used for immunofluorescence labeling only works on live cells, a contactin-GFP chimera was used in place of contactin to allow visualizing the intracellular pool of this protein (Supplementary Figure S2). The GFP sequence was inserted at the carboxy terminus of the GPI-anchor sequence of contactin. Live cell immunostaining using anti-GFP antibodies indicated that GFP is extracellular (Figure S2B). Contactin-GFP was detected as a

Figure 5 (cont). PGY (lane 5–10). Immunoblots were revealed using anti-paranodin, anti-caspr2, or anti-calnexin antiserum in the lysates (L) and immunoprecipitates (ip). Calnexin is coimmunoprecipitated with paranodin (lane 2) and with pndΔPGY2 (lane 4). Calnexin does not associate with caspr2 (lane 6), is detected at low levels in the pnd-caspr2C immune precipitate (lane 8), and is not detected in the caspr2-PGY immune precipitate (lane 10).

Figure 7. Paranodin requires COPI vesicles for export but does not accumulate in the Golgi after temperature block. (A–C) N2a cells transfected with the dominant negative HA-tagged Arf1(Q71L) were fixed 14 h after transfection. (A) Paranodin coexpressed with contactin (red) is recruited with Arf1(Q71L) (blue) in the Golgi (arrowheads). (B) In cells only transfected with Arf1(Q71L), the ER resident chaperone BiP (red) is recruited for a part in the Golgi with Arf1(Q71L) (green), due to inhibition of the COPI-mediated retrieval from the Golgi to the ER. (C) In contrast, paranodin expressed alone (red) is retained in the ER of cells cotransfected with Arf1(Q71L) (green), indicating that its retention does not result from Golgi-to-ER retrieval. (D–F) N2a cells transfected with contactin-GFP (D) paranodin and contactin-GFP (E), or pnd-caspr2C (F) were incubated during 4 h at 25°C before fixation with methanol 14 h after transfection. After temperature block, contactin-GFP (D, green) strongly accumulates in the Golgi apparatus and colocalizes with the 58K Golgi marker in red (arrowheads). In contrast, in N2a cells coexpressing contactin-GFP (green) and paranodin (red; indicated by asterisks in E) both molecules are distributed in the ER or at the cell membrane but do not colocalize with the 58K Golgi marker in blue (E). Contactin-GFP is concentrated in the Golgi apparatus (arrowheads in E) in a cell that does not coexpress paranodin. Pnd-caspr2C (F, green) is detected in the Golgi apparatus of some of the transfected N2a cells after cold block and colocalized with the 58K Golgi marker in red. Bar, 30 μ m. (G) Quantitative analyses of the results presented in A. The percentage of cells with plasma membrane expression of paranodin coexpressed with contactin is significantly reduced by coexpression of Arf1(Q71L) when compared with control conditions (ANOVA, $p < 0.01$). (H) Quantitative analyses of the results presented in E. Cells were incubated at 37°C for 10 h after transfection (T) and were then incubated for an additional 4-h period at 25°C (control conditions) or were shifted to 25°C. The percentage of cells with plasma membrane expression of paranodin is significantly reduced after incubation at 25°C when compared with control conditions (ANOVA, $p < 0.01$). Means $\pm$ SEM of three independent experiments. More than 100 cells were analyzed in each experiment.

band of 170 kDa by Western blot using anti-GFP or anti-contactin antibodies (Supplementary Figure S2A). When coexpressed with contactin-GFP, paranodin associated with contactin-GFP (Supplementary Figure S2A) and was targeted to the cell membrane as with native contactin (Supplementary Figure S2C). Therefore, contactin-GFP was appropriate to analyze the trafficking of the paranodin complex.

All the recombinant proteins were detected in the ER 4 h after transfection, whereas they were delivered to the cell membrane with different kinetics. Caspr2 and contactin-GFP were detected at the cell membrane 6 h after transfection, whereas paranodin cotransfected with contactin and pnd-

caspr2C reached the cell membrane only 12 h after transfection (Supplementary Table S1). Thus, paranodin and pnd-caspr2C appeared to require a long-lasting processing (6–8 h) before their delivery to the cell membrane compared with caspr2. Strikingly, the pnd Δ PGY2 mutant required an even much longer delay for maturation in the ER because it was only detected at the plasma membrane 28 h after transfection (Supplementary Table S1).

To further analyze the trafficking of paranodin and contactin to the cell membrane, we used temperature blockade at 25°C that prevents exit from the *trans*-Golgi network and induces accumulation of secreted proteins in the Golgi ap-

Figure 8. NF155-Fc binds contactin bearing high-mannose N-glycans. (A–D) N2a cells transfected with contactin were untreated (A and C) or incubated during 18 h with tunicamycin (B and D). Cells were incubated with NF155-Fc (A and B) or NrCAM-Fc (C and D) at an estimated concentration of 20 μ g/ml during 30 min and immunostained using anti-Human Fc antibodies. Tunicamycin treatment prevented NF155-Fc binding. Bar, 40 μ m. (E) N2a cells transfected with contactin and paranodin were incubated with NF155-Fc and anti-Human Fc antibodies, fixed, and permeabilized before immunostaining for paranodin. Note that NF155-Fc binding sites colocalize with paranodin. Bar, 20 μ m. (F) Schematic representation of the different N-glycan structures anchored at Asn (N) residues in the polypeptide chains and produced by the parental and mutant CHO cell lines. N-acetylglucosamine is abbreviated Gluc-Nac in the legend. (G) Contactin expressed in transfected parental and glycosylation mutant CHO cells was analyzed by SDS-PAGE and immunoblotting. In parental CHO cells, contactin migrates as a doublet of 142 and 135 kDa. By comparison, a single glycosylated variant of contactin is observed in each of the Lec10, Lec1, and Lec23 mutant lines with different apparent molecular weights. An unspecific band indicated with an asterisk at 120 kDa is also detected in untransfected CHO cells. (H) Parental CHO cells and the Lec10, Lec1, and Lec23 mutant lines were transfected with contactin. Immunostaining for contactin on live cells indicates that the different glycoforms of contactin are expressed at the cell surface. (I) Parental CHO cells were transfected with contactin (CHO) or cotransfected with contactin and paranodin (CHO + pnd). Lec10, Lec1, and Lec23 mutant lines were transfected with contactin. Cells were incubated with NF155-Fc at an estimated concentration of 10 μ g/ml during 30 min and immunostained using Texas red-conjugated anti-Fc antibodies. All images were collected using identical confocal settings. Bar, 20 μ m. (J) Quantitative analysis of the results presented in I. NF155-Fc binding is expressed in arbitrary units corresponding to the mean of fluorescence per cell. More than 40 cells were analyzed in each experiment. NF155-Fc binding significantly increased in the Lec1 and Lec23 mutants when compared with parental CHO cells. Similarly, NF155-Fc binding significantly increased in CHO cells cotransfected with paranodin when compared with CHO cells expressing contactin alone (ANOVA, $p < 0.01$). (K) CHO cells cotransfected with paranodin and contactin were lysed with 1% NP-40. The lysate was untreated (–; lane 1), or incubated with Endo H (H; lane 2), and immunoblotting was performed with both anti-paranodin and anti-contactin antisera. Paranodin (asterisk) detected at 180 kDa is Endo H-sensitive in the lysate. The 142-kDa glycoform of contactin (○) is Endo H-resistant, whereas the 135 kDa glycoform of contactin (●) is Endo H-sensitive in the lysate. The lysate was incubated with NF155-Fc linked to protein A-Sepharose and, after washing, bound proteins were eluted and incubated with Endo H (H; lane 3). Paranodin (asterisk) and contactin (●), eluted from the NF155-Fc protein A-Sepharose, are Endo H-sensitive.

paratus (Matlin and Simons, 1983). Temperature blocks were applied for a period of 4 h just before the onset of surface delivery of transfected proteins. Caspr2 (not shown) or contactin-GFP strongly accumulated in the Golgi when cells were incubated during 4 h at 25°C, as shown by its colocalization with the Golgi marker 58K (Figure 7D). In contrast, we never observed any colocalization of paranodin with 58K in cells cotransfected with contactin-GFP and incubated at 25°C for a period of 4 or 8 h (Figure 7E). Quantification of the data showed that the temperature shift inhibited for a part the cell membrane expression of paranodin (Figure 7H). Interestingly, contactin-GFP associated with

paranodin (i.e., in cells coexpressing both proteins) did not accumulate in the Golgi after incubation at 25°C (Figure 7E, asterisks), whereas it colocalized with 58K in the cells expressing contactin-GFP alone (Figure 7E, arrowheads). Thus, paranodin appeared to prevent the default exocytic pathway contactin-GFP. In contrast with paranodin, the pnd-caspr2C chimera was colocalized with the 58K Golgi marker after incubation at 25°C in some of the cells (Figure 7F, arrowheads). This result is in agreement with the existence of an Endo H-resistant pool of pnd-caspr2C (Figure 6B), indicating that the chimera lacking PGY traffics to the cell surface via the classical Golgi pathway.

Altogether these results indicate that paranodin trafficking is unusually long and diverges from that of standard Golgi-processed proteins.

Contactin with High-Mannose N-Glycans Strongly Associates with NF155

Association with paranodin allows the cell surface expression of a low M_r form of contactin with high-mannose ER-type N-glycans (Rios *et al.*, 2000; Bonnon *et al.*, 2003; Gollan *et al.*, 2003). As previously shown using cell surface biotinylation assay, both paranodin and contactin are endo-H-sensitive when expressed in complex at the cell surface (Bonnon *et al.*, 2003). It is still unclear if and how this glycoform of contactin binds NF155, which is expressed by the glial paranodal loops (Tait *et al.*, 2000). An NF155-Fc chimera specifically binds CHO cells coexpressing contactin and paranodin and precipitates these proteins from brain lysates (Charles *et al.*, 2002). On the other hand, it has been reported that the coexpression of paranodin with contactin in COS-7 cells prevented NF155-Fc binding to contactin, and NF155-Fc was associated in *cis* with the high M_r form of contactin in HEK-293 cells (Gollan *et al.*, 2003). Because these contradictory results suggest a role of paranodin-controlled glycosylation of contactin, we first examined whether the binding of NF155 on contactin may depend on its N-glycan residues.

Tunicamycin treatment did not modify the cell surface expression of contactin in transfected N2a cells (Bonnon *et al.*, 2003), whereas it completely prevented binding of NF155-Fc (20 μ g/ml) (Figure 8, A and B). Binding of NrCAM-Fc, another ligand of contactin, was unaffected (Figure 8, C and D). We then used mutant CHO lines affected in the processing of N-linked carbohydrates (Stanley and Ioffe, 1995) to test whether NF155 may interact with contactin bearing either high-mannose residues or complex oligosaccharide chains. The Lec1 line mutated for the N-acetylglucosamine transferase I produces N-glycans with the high-mannose configuration $\text{Man}_5\text{GlcNAc}_2$, the Lec23 line is mutated for the α -glucosidase I preventing trimming of $\text{Glc}_3\text{Man}_{7-9}\text{GlcNAc}_2$ N-glycans and the Lec10 line overexpressing the N-acetylglucosamine transferase III produces bisected complex N-glycans (Figure 8F). Western blot analyses indicated that each glycosylation mutant produced different forms of contactin, indicating an altered carbohydrate content (Figure 8G). Cell surface expression of contactin was comparable in transfected parental and mutant CHO cells as estimated by live cells immunostaining with anti-contactin antibody 24 (Figure 8H). Contactin with complex N-glycans when expressed by the parental or Lec10 CHO lines faintly bound NF155-Fc (10 μ g/ml; Figure 8, I and J). In contrast, both the Lec1 and Lec23 cell lines expressing contactin bearing high-mannose glycans strongly bound NF155-Fc (Figure 8, I and J). Cotransfection of paranodin with contactin in the Lec1 and Lec23 lines did not prevent the strong NF155 binding (not shown). Parental CHO cells coexpressing contactin and paranodin at their cell surface with high-mannose glycans strongly bound NF155-Fc (Figure 8, I and J). Quantitative analyses indicated that binding of NF155 was significantly increased on cells with high-mannose contactin when compared with parental CHO cells expressing complex N-glycan contactin (Figure 8J). In addition, both paranodin and contactin from CHO cell lysate were affinity-purified using NF155-Fc protein A-Sepharose. Paranodin and contactin in complex with NF155-Fc displayed Endo H-sensitivity (Figure 8K). Finally, NF155-Fc binding sites were colocalized with paranodin in N2a cells cotransfected with contactin and paranodin (Figure 8E). Altogether, these results show that the paranodin-controlled glycosylation of contactin induces strong

binding of neurofascin-155 and allows the three adhesion glycoproteins to form a tripartite complex.

DISCUSSION

In the present study, we provide new insights into mechanisms that ensure the association of paranodin with contactin and trigger the export of the complex to the plasma membrane. Using sequence comparison and domain swapping with the related protein caspr2, we identified a PGY motif in the ectodomain of paranodin responsible for its ER retention. Deletion of the PGY region results in the cell surface targeting of paranodin in the absence of contactin. Reciprocally, insertion of PGY into a topologically similar location in caspr2 or in NrCAM is sufficient to block these proteins in the ER. This sequence acts as a signal that compels the protein to associate with contactin and to pass the calnexin/calreticulin checkpoint before ER export. Contactin associated in complex with paranodin is expressed at the cell surface with mannose-rich Endo H-sensitive N-glycans and strongly binds NF155, the glial partner of contactin at paranodes.

The ER Retention Motif of Paranodin Resides in Its Ectodomain

ER retention is a widely used mechanism that prevents the cell surface delivery of unassembled subunits of membrane channels or receptors. Many ER retention signals are cytoplasmic motifs such as the dibasic KKXX or arginine-based RXR motifs, whereas luminal retention motifs have not been identified, with the exception of the well-characterized KDEL signal that retains soluble resident proteins in the ER (Ellgaard and Helenius, 2003). Here, we found that the ER retention motif of paranodin has an extracellular location between the EGF-2 and the Laminin G (LNG)-4 domains. It consists of a proline-rich sequence of ~ 50 residues with 10 imperfect repeats of the PGY(X)₁₋₂ motif where X is any amino acid. Motifs with PGY repeats of unknown function are encountered in a variety of prokaryotic and eukaryotic proteins, among which some are secreted. The PGY motif is not found in the other members of the neurexin IV/caspr/paranodin family, including caspr2, caspr3 and caspr4 (Spiegel *et al.*, 2002). In addition, neurexin IV, the only *Drosophila* ortholog of these proteins, does not contain PGY and does not display any ER retention (Faivre-Sarrailh *et al.*, 2004). Thus, the PGY motif appears to be a specific characteristic of paranodin that accounts for its unusual trafficking and exclusive cellular localization.

The PGY Motif May Impose a Conformational Processing of Paranodin before Export

Proline-rich sequences with repeated motifs often correspond to extended protein regions which are in an unfolded state and undergo multiple protein-protein interactions (Williamson, 1994; Rath *et al.*, 2005). Interestingly, structural prediction studies suggest that the PGY region may adopt a stable organized β -sheet structure. Because contactin does not interact directly with PGY, an attractive hypothesis would be that contactin might act as a chaperone and induce a disorder-to-order transition of the PGY-rich sequence, allowing its transport to the cell surface (Figure 9A).

A key step in the cell surface delivery of paranodin is linked to the quality control by the lectin chaperones calnexin/calreticulin in the ER (Bonnon *et al.*, 2003). The lectin chaperones bind membrane glycoproteins via direct interaction with the N-terminal luminal domain and/or N-linked oligosaccharide chains (Ou *et al.*, 1993) and retain unfolded

Figure 9. Model of cooperative interaction between contactin and paranodin. (A) The inset shows the 3-D structure of the PGY motif analyzed by the Rosetta prediction method. The PGY motif (aa1027–1084) is organized with four β -sheets. The PGY motif might display a dynamic structure when paranodin is expressed alone and blocked in the ER. Association with contactin might stabilize the PGY-rich sequence into an organized β -sheet structure, allowing export of paranodin to the plasma membrane (PM). (B) The presence of PGY repeats governs an unconventional processing of paranodin and contactin, which are expressed at the cell surface with ER-type mannose-rich N-glycans. The high-mannose glycoform of contactin displays strong binding activity for NF155, allowing the formation of axo-glial adhesive contacts at paranodes. In contrast, the contactin glycoform expressed at the node, contains complex N-glycans and thereby displays low affinity for NF155.

proteins in the ER until they acquire a correct tridimensional arrangement (Schrage *et al.*, 2003). The PGY repeats do not mediate direct interaction with calnexin but may act as a conformational signal that compels chaperoning of paranodin through the calnexin/calreticulin cycle. Indeed, deletion of PGY in pnd Δ PGY2 and pnd-caspr2C allows the mutant proteins to be expressed at the cell surface bypassing the calnexin/calreticulin cycle. Conversely, PGY inserted into caspr2 induces ER retention and chaperoning for a part by calnexin/calreticulin. Association with contactin powerfully induces the cell surface expression of paranodin, whereas it does not improve cell surface delivery of pnd Δ PGY2 and pnd-caspr2C. Therefore, we can propose that PGY may be required to generate the fully stable conformation of paranodin through the chaperoning by contactin and calnexin/calreticulin cycle. Interestingly, even in the absence of PGY, the cell surface delivery of pnd Δ PGY2 or pnd-caspr2C required a long-lasting processing in the ER and was prevented in presence of tunicamycin. This result underscores the crucial role of N-glycans in generating a permissive conformation for the cell surface trafficking of paranodin.

Long-lasting Processing of Paranodin before Export to the Cell Surface

We have previously reported that the complex of paranodin and contactin is expressed at the plasma membrane with ER-type Endo H-sensitive carbohydrates and may traffic to the cell surface through an unconventional pathway that bypass the Golgi apparatus (Bonnon *et al.*, 2003). Recent studies indicate that few transmembrane proteins may traffic to the plasma membrane via a Golgi-independent pathway, including the protein phosphatase CD45 (Baldwin and Ostergaard, 2002) and the epithelial sodium channel (Hughey *et al.*, 2004). The precise nature of this pathway remains to be elucidated. In the present study, we have reevaluated the possibility of a Golgi-independent pathway for paranodal proteins using the dominant negative mutant

of Arf1, Arf1(Q71L), and temperature blockade of export. The cell surface expression of paranodin associated with contactin was prevented by Arf1(Q71L) revealing its dependence on COPI vesicles. However, the role of COPI vesicles is complex since it is implicated in both anterograde and retrograde transport from Golgi to the ER (Rabouille and Klumperman, 2005). On the other hand, temperature blockade of paranodin export did not induce any accumulation of the protein in the Golgi. This unconventional ER processing of paranodin associated with contactin takes a long time (over 8 h) and may require recycling from the Golgi to the ER before exit of the complex to the cell surface.

Processing of Paranodin and Contactin with High-Mannose N-Glycans May Induce Their Selective Association with Glial NF155 at Paranodes

The unconventional processing of paranodin and contactin leads to the expression of these glycoproteins with mannose-rich N-glycans at paranodes. By contrast, the contactin isoform expressed at the node may bear complex N-glycans (Rios *et al.*, 2000). NF155, which is selectively expressed by the paranodal glial loops is required for the restricted positioning of contactin and paranodin along the myelinated axons at paranodes (Sherman *et al.*, 2005). Neurofascin and contactin, as the other members of the IgCAM family, show a broad activity of binding. Therefore, the selective association of these IgCAMs at paranodes may require a fine tuning. The generation of specific splice variants, such as the NF155 isoform expressed at paranodes, provides a way to regulate the binding activities of neurofascin (Volkmer *et al.*, 1998). On the other hand, we show here a crucial role of paranodin in controlling the differential maturation of N-linked carbohydrates of contactin, which in turn regulates its association with NF155. The use of N-glycosylation mutant cell lines provides the direct demonstration that NF155 strongly binds the low M_r form of contactin bearing high-mannose N-glycans. These data challenge a previous study

of Gollan *et al.* (2003) indicating that NF155 may only bind the high M_r form of contactin. NF155 may directly bind the oligosaccharide chains of contactin. Alternatively, the presence of high-mannose N-glycans on contactin may support an optimal conformation of the polypeptide for the binding of NF155.

Thus, this study reveals the structural basis for the processing differences between caspr2 and paranodin, two proteins, which in spite of a high degree of sequence homology, have distinct localization and functions. The PGY motif, specific to paranodin, forces this protein to follow a specific maturation pathway, which can be completed only in the presence of contactin. Reciprocally, contactin associated with paranodin is processed with mannose-rich N-glycans to the cell surface. This may be a critical mechanism for the binding of the glial partner NF155 at the paranodal junctions, for the generation of specialized subdomains at nodes of Ranvier and thus for the positioning of voltage-gated ion channels that are essential for an effective nerve conduction.

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