

Association of TAG-1 with Caspr2 is essential for the molecular organization of juxtaparanodal regions of myelinated fibers

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Myelination results in a highly segregated distribution of axonal membrane proteins at nodes of Ranvier. Here, we show the role in this process of TAG-1, a glycosyl-phosphatidyl-inositol-anchored cell adhesion molecule. In the absence of TAG-1, axonal Caspr2 did not accumulate at juxtaparanodes, and the normal enrichment of *shaker*-type K⁺ channels in these regions was severely disrupted, in the central and peripheral nervous systems. In contrast, the localization of protein 4.1B, an axoplasmic

partner of Caspr2, was only moderately altered. TAG-1, which is expressed in both neurons and glia, was able to associate in cis with Caspr2 and in trans with itself. Thus, a tripartite intercellular protein complex, comprised of these two proteins, appears critical for axo–glial contacts at juxtaparanodes. This complex is analogous to that described previously at paranodes, suggesting that similar molecules are crucial for different types of axo–glial interactions.

Introduction

Myelinated axons in the central and peripheral nervous systems (PNSs) are organized into distinct domains as a consequence of their interactions with glial cells (Vabnick et al., 1997; Kaplan et al., 1997, 2001; Dupree et al., 1999; Mathis et al., 2001; Arroyo et al., 2002). Paranodal junctions separate the nodes of Ranvier from internodal regions. At their level, the

extremities of compact myelin wraps expand in a series of cytoplasm-filled loops, which adhere to the neuronal plasma membrane, or axolemma, forming septate-like axo–glial junctions. Several adhesion proteins have been localized at these junctions (for reviews see Peles and Salzer, 2000; Girault and Peles, 2002; Scherer and Arroyo, 2002). In the paranodal axolemma, paranodin/Caspr, a member of the neurexin IV-caspr-paranodin (NCP) group of the neurexin superfamily, is associated with contactin/F3, a cell adhesion molecule of

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Key words: TAG-1/axonin-1/contactin-2; paranodin/Caspr/NCP-1; potassium channels; protein 4.1B; nodes of Ranvier

Abbreviations used in this paper: CMAP, compound muscle action potential; CNS, central nervous system; GPI, glycosylphosphatidylinositol; IB, immunoblot; IF, immunofluorescence; IgSF, Ig superfamily; IP, immunoprecipitation; IR, immunoreactivity; MAG, myelin-associated glycoprotein; NCP, neurexin IV-caspr-paranodin; P8, postnatal day 8; PNS, peripheral nervous system.

the immunoglobulin superfamily (IgSF; Einheber et al., 1997; Menegoz et al., 1997; Rios et al., 2000). Contactin/F3, a glycosylphosphatidylinositol (GPI)-linked protein, is necessary for recruiting paranodin/Caspr to the plasma membrane and for its targeting to the paranodes (Faivre-Sarrailh et al. 2000; Rios et al., 2000; Boyle et al., 2001). The complex formed by these two proteins interacts with the 155-kD isoform of neurofascin (NF155), another IgSF protein expressed by glial cells and concentrated in paranodal loops (Tait et al., 2000; Charles et al., 2002). In the absence of either paranodin/Caspr or contactin/F3 paranodal junctions are severely altered and lose their septate-like appearance (Bhat et al., 2001; Boyle et al., 2001). Thus, these three proteins are thought to form an intercellular complex linking the glial plasma membrane to the axolemma and are necessary for the formation of septa (for review see Girault and Peles, 2002). These transmembrane intercellular complexes interact with axoplasmic proteins through the cytoplasmic region of paranodin/Caspr which encompasses a glycoprotein C-neurexin IV-paranodin-binding motif for the protein 4.1 family (Menegoz et al., 1997; Girault et al., 1998). Protein 4.1B has been recently identified as a major cytoplasmic partner of paranodin/Caspr at paranodes and may provide an anchor to the actin/spectrin based axonal cytoskeleton (Ohara et al., 2000; Gollan et al., 2002; Denisenko-Nehrbass et al., 2003b).

The *shaker*-type delayed-rectifier potassium channels subunits, Kv1.1 and Kv1.2, are clustered in juxtaparanodal regions (Wang et al., 1993). Although their function remains largely unknown, the localization of K⁺ channels at juxtaparanodes may stabilize conduction and maintain the internodal resting potential (Zhou et al., 1998; Vabnick et al., 1999). Caspr2, a member of the NCP family, closely related to paranodin/Caspr (45% amino acid identity), is also enriched in the juxtaparanodal axolemma (Poliak et al., 1999). Caspr2 can be coimmunoprecipitated with K⁺ channels, and their interaction may involve a protein with PDZ domains (Poliak et al., 1999), yet to be identified (Rasband et al., 2002). Caspr2 cytoplasmic domain encompasses a glycoprotein C-neurexin IV-paranodin motif and associates in vitro and in vivo with protein 4.1B that is also present at juxtaparanodes (Denisenko-Nehrbass et al., 2003b). However, it is not known whether Caspr2 is associated with other axonal membrane protein(s) and whether it interacts with glial proteins.

Recently, TAG-1, an IgSF protein closely related to contactin/F3 (50% amino acid identity) was found to be enriched in juxtaparanodal regions of central nervous system (CNS) and PNS myelinated fibers (Traka et al., 2002). TAG-1, which exists in neurons as a GPI-anchored protein and as a secreted form, was originally described as a protein transiently expressed in axons during development of rodents (Dodd et al., 1988; Furley et al., 1990; Karagogeos et al., 1991). The protein is also expressed by oligodendrocytes and Schwann cells, the myelinating glial cells of the CNS and PNS, respectively (Traka et al., 2002). TAG-1 immunoreactivity (IR) is colocalized with Kv1.1 channels and Caspr2 (Traka et al., 2002), but it is not known whether and how these proteins interact.

Here, we have investigated the function of TAG-1 in axo-glial contacts by analyzing the phenotype of TAG-1-defi-

cient mice and observed that the distribution of juxtaparanodal proteins, Caspr2 and K⁺ channels, was severely altered in the CNS and PNS of the mutant mice. We have also examined the interactions between TAG-1 and Caspr2 in brain and in transfected cells, and found that TAG-1 and Caspr2 are able to associate. Thus, this paper reveals that proteins of the NCP family and GPI-anchored IgSF cell adhesion molecules form conserved complexes important for the organization of markedly distinct axonal domains in nodal regions of myelinated fibers.

Results

The basic ultrastructural organization of myelinated fibers and nerve function are preserved in TAG-1 mutant mice

TAG-1-deficient mice survive and reproduce normally (Fukamauchi et al., 2001). Although mutant mice display a greater sensitivity to pro-convulsant stimuli and a marked elevation of adenosine A1 receptors in the hippocampus, morphological analysis of the cerebellum, spinal cord, and hippocampus of these mice did not reveal gross abnormalities (Fukamauchi et al., 2001). Because TAG-1 is also expressed in oligodendrocytes, we examined whether the absence of TAG-1 altered the ultrastructural organization of myelinated axons by electron microscopy. In ultrathin transverse spinal cord sections, myelin sheath thickness and compaction were similar in myelinated fibers of the ventral spinal cord region of wild-type (Fig. 1 A) and TAG-1 mutant mice (Fig. 1 B). The nodal, paranodal, and juxtaparanodal regions appeared properly organized in spinal cord sections of both wild-type (Fig. 1 C) and mutant animals (Fig. 1 D). Finally, the transverse bands, the hallmarks of normal axo-glial junctions, were normally present in both genotypes (Fig. 1, E and F, arrowheads). These results show that TAG-1 expression is not required for myelin sheath formation and structural organization of distinct axonal domains.

We also examined whether the absence of TAG-1 would result in abnormal nerve function by performing basic electrophysiological studies on sciatic nerves of 2-mo-old wild-type and mutant mice. Compound muscle action potentials (CMAPs) recorded after distal and proximal stimulation of sciatic nerves, and F waves, which represent late responses from antidromically activated motor neurons, were similar in both genotypes (Fig. 1, G and H). There was no difference in CMAP amplitudes, distal latencies or conduction velocities between wild-type, heterozygous, and homozygous TAG-1 mutant nerves (Table I). In addition, there was no evidence of partial conduction block or temporal dispersion that would indicate the presence of a demyelinating neuropathy. Altogether these findings indicated the absence of major morphological and functional deficit in the myelinated fibers of TAG-1 knockout mice.

Distribution of juxtaparanodal proteins is dramatically altered in the CNS of TAG-1-deficient mice

We examined whether the absence of TAG-1 altered axo-glial interactions at the molecular level by studying the localization of markers of nodal regions by immunofluorescence

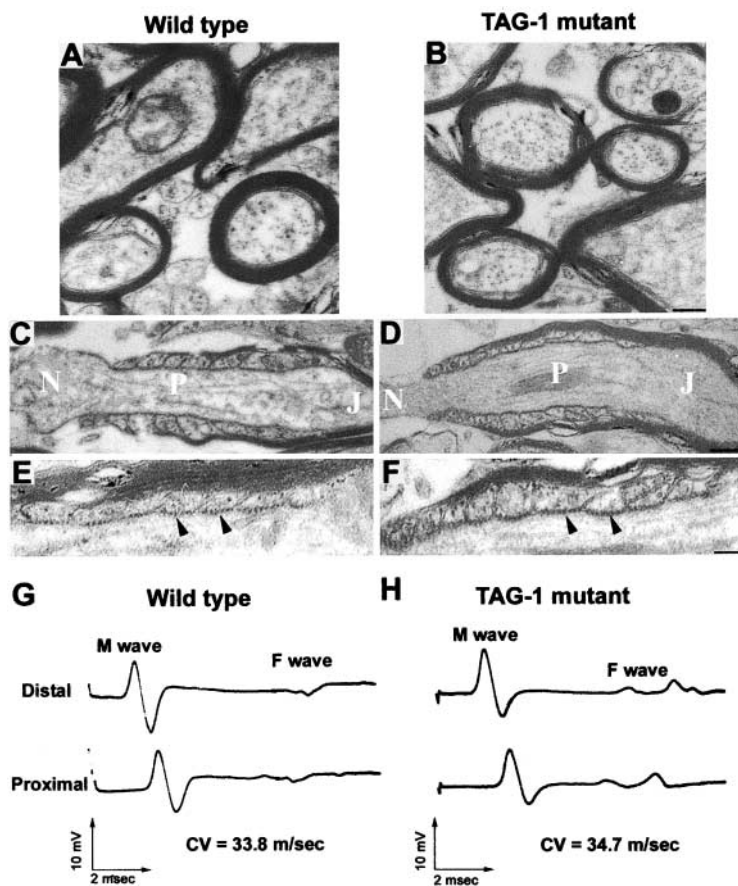


Figure 1. Ultrastructural organization of myelinated fibers and nerve function in TAG-1 mutant mice.

(A–F) Ultrastructural organization of myelinated fibers. Myelin sheath thickness and compaction were similar in myelinated fibers of the ventral spinal cord region of 3-mo-old (A) wild-type and (B) TAG-1 mutant mice. The nodal (N), paranodal (P), and juxtaparanodal (J) regions appeared properly organized in longitudinal sections of wild-type (C) and mutant animals (D). In the paranodal region, the transverse bands (arrowheads) were normally present in both genotypes (E, wild type; F, mutant). Bars: (A and B) 0.1 μm ; (C and D) 0.5 μm ; and (E and F) 0.3 μm . (G and H) Electrophysiological studies of sciatic nerves. CMAPs and F waves were recorded after distal and proximal stimulation of sciatic nerves of 2-mo-old (G) wild-type and (H) TAG-1 mutant mice. There were no differences in the waveform, latencies and amplitudes of CMAPs between both genotypes.

(IF) and laser confocal microscopy in optic nerve sections of wild-type and TAG-1-deficient mice. Double labeling for sodium channels and paranodin/Caspr in wild-type (Fig. 2 A) and mutant mice (Fig. 2 B) demonstrated normal clustering of these proteins in the nodal and paranodal regions, respectively, in both genotypes. We analyzed the organization of the juxtaparanodal regions of the TAG-1 mutant optic nerves by examining the expression of Caspr2 and Kv1.1 potassium channels subunits. As expected, in wild-type animals, Caspr2 was detected at juxtaparanodes in reference to the nodal sodium channels (Fig. 2 C), whereas in mutant mice, no enrichment of Caspr2 IR was detectable (Fig. 2 D). K^+ channels distribution was also dramatically altered in mutant fibers because the overall staining for Kv1.1 was decreased and appeared to occupy much smaller areas than in wild-type animals (Fig. 2, E and F). Kv1.1-IR was detected

only at the vicinity of paranodes in almost all the sites examined in the mutant optic nerves (Fig. 2 F, arrows). Similar alterations of Caspr2 and Kv1.1 channel distribution, contrasting with unchanged nodal and paranodal markers, were observed in spinal cord (unpublished data), indicating that TAG-1 plays a general role in the organization of axo–glial juxtaparanodal contacts in the CNS.

The dramatic alterations of Caspr2 and Kv1.1-IR in juxtaparanodal regions raised the possibility that TAG-1 might control either their expression levels or their enrichment in these regions. To address this question we measured the levels of these proteins by immunoblot (IB) of optic nerve extracts. In the mutants, as expected, no TAG-1 immunoreactive band was observed (Fig. 3 A), whereas Caspr2 levels were virtually unchanged (Fig. 3 B). Kv1.1 antibodies revealed a doublet (Fig. 3 C), which may correspond to the 86-kD mature,

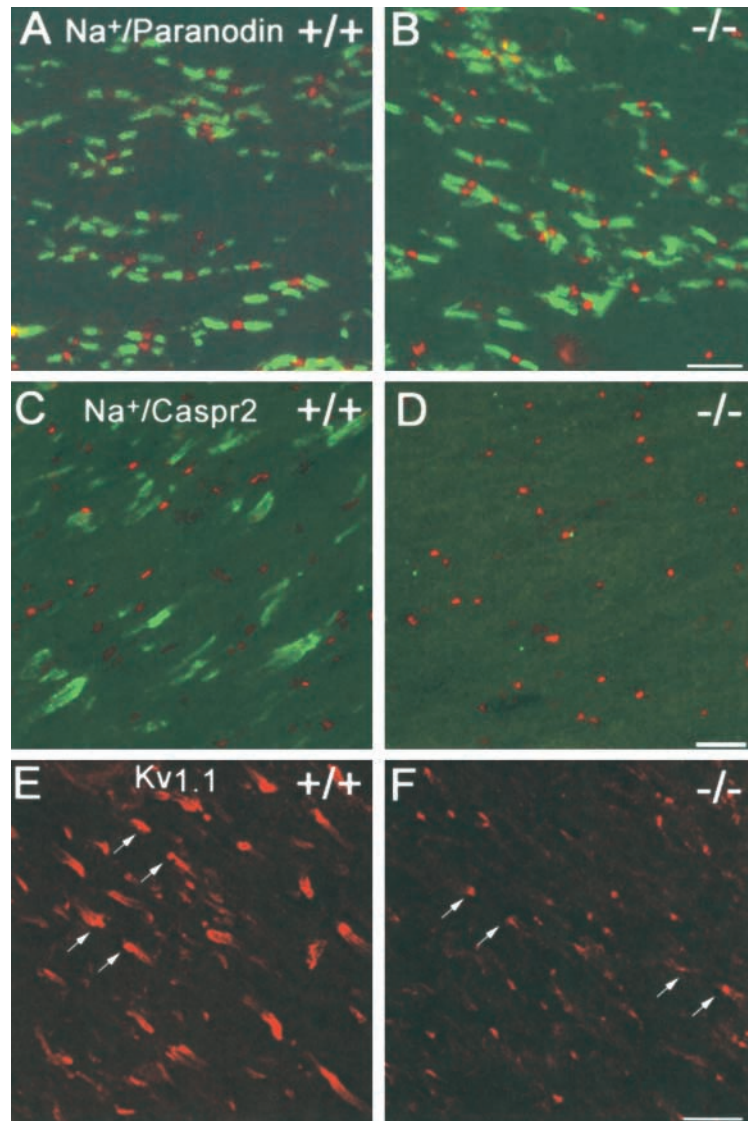
Table I. Parameters of compound action potentials from wild-type and TAG-1 mutant sciatic nerves

	Units	(+/+) <i>n</i> = 9	(+/-) <i>n</i> = 5	(-/-) <i>n</i> = 11
DL	ms	1.14 \pm 0.19	1.18 \pm 0.15	1.17 \pm 0.16
PL	ms	1.82 \pm 0.18	1.78 \pm 0.15	1.83 \pm 0.20
CV	m/s	34.02 \pm 3.64	35.64 \pm 2.83	35.23 \pm 3.61
AMP (D)	mV	11.51 \pm 4.54	11.76 \pm 3.46	14.90 \pm 3.03
AMP (P)	mV	10.11 \pm 3.51	10.78 \pm 2.65	13.06 \pm 2.99

Distal latencies (DL), proximal latencies (PL), conduction velocities (CV), distal (D), and proximal (P) CMAP amplitudes (AMP) were measured in sciatic nerves of wild-type (+/+), and heterozygous (+/-) or homozygous (-/-) mutant 2-mo-old mice.

^a*P* > 0.05 ANOVA for all parameters.

Figure 2. Distribution of specific proteins in myelinated optic nerves of TAG-1 mutant mice. Localization of molecular components of nodes, paranodes, and juxta-paranodes in optic nerve sections of 2-mo-old (A, C, and E) wild-type (+/+) and (B, D, and F) TAG-1 mutant (-/-) mice. Sodium channels (red) and paranodin/Caspr (green) were normally clustered in the nodal and paranodal regions, respectively, in both (A) wild-type and (B) TAG-1-deficient mice. Caspr2-IR (green) was normally detected in the juxtapanodal regions, in reference to the nodal sodium channels (red) in (C) wild-type animals, whereas it was not visible in (D) TAG-1 mutant mice. Kv1.1-IR was dramatically altered in mutant mice (F, arrows), as compared with wild-type animals (E, arrows). In TAG-1 mutant mice, Kv1.1 labeling was markedly decreased and was mostly restricted to a small area in contact with (F) paranodes. Bars: (A-F) 5 μ m.

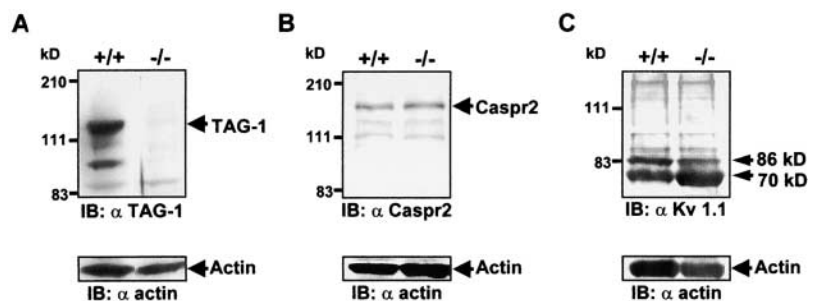


and 70-kD immature forms of these channels (Manganas and Trimmer, 2000). In mutant mice, the lower immunoreactive band was markedly increased, whereas the upper band was not significantly altered (Fig. 3 C). Thus, the biochemical measurements demonstrated that the dramatic alterations in Caspr2 and Kv1.1 channels in TAG-1 mutant mice did not result from defects in their expression but rather from the lack of concentration of these proteins in juxtapanodal regions, presumably below IF detection levels.

Juxtapanodal markers are severely altered in the PNS of TAG-1 mutant mice

To determine whether TAG-1 was also critical for the molecular organization of peripheral nerve fibers, we examined the localization of markers of the nodal regions in sciatic nerves of TAG-1 knockout mice and wild-type littermates (Fig. 4). In wild-type animals, TAG-1-IR was enriched in juxtapanodal regions, adjacent to paranodes, labeled with

Figure 3. Expression levels of juxtapanodal proteins in central myelinated fibers of TAG-1 knockout mice. Expression levels of (A) TAG-1, (B) Caspr2, and (C) Kv1.1 potassium channels in 2-mo-old wild-type (+/+) and TAG-1 mutant mice (-/-) were examined by IB analysis of optic nerve extracts (top). Protein levels were quantified (three mice in each group) using actin (bottom) in each sample for normalization: Caspr2 levels in mutant mice were $84 \pm 2\%$ of wild-type (mean \pm SD); Kv1.1 86-kD band; $129 \pm 22\%$; and 70-kD band $193 \pm 21\%$. The position of molecular mass markers (kD) is indicated.



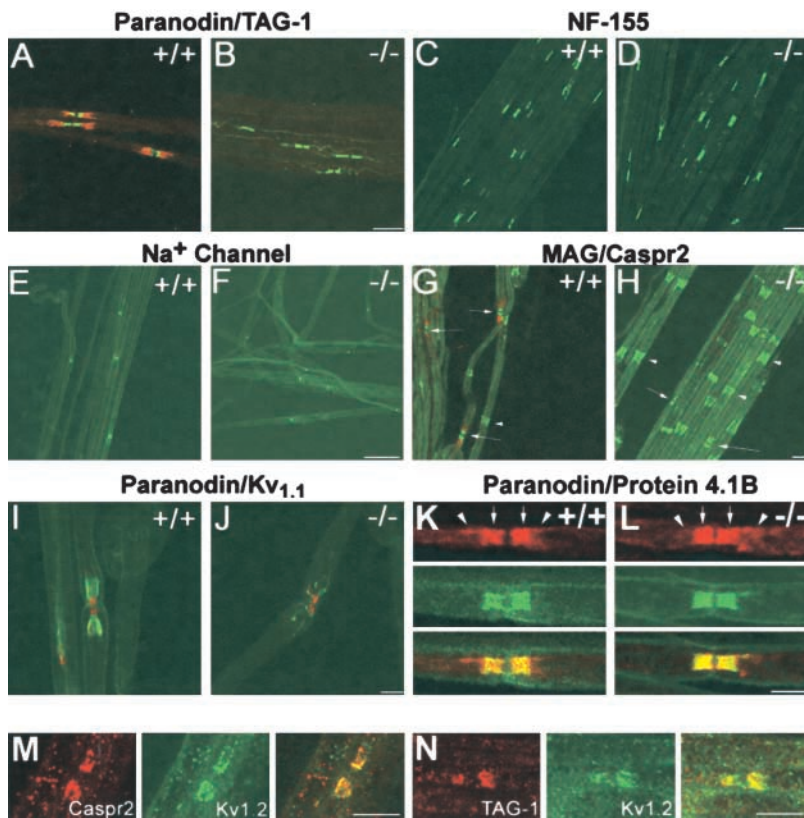


Figure 4. Distribution of specific proteins in sciatic nerves nodal regions of TAG-1 mutants and during development. (A–L) Localization of molecular components of nodes, paranodes, and juxtaparanodes in teased sciatic nerve fibers of 2-mo-old (A, C, E, G, I, and K) wild-type (+/+) and (B, D, F, H, J, and L) TAG-1 mutant (–/–) mice: (A and B) TAG-1 (red) and paranodin/Caspr (green); (C and D) NF155; (E and F) sodium channels; (G and H) MAG (green, paranodal regions, arrows; Schmidt-Lanterman incisures, arrowheads) and Caspr2 (red); (I and J) Kv1.1 potassium channels (green) and Caspr/paranodin (red); (K and L) Protein 4.1B (red) and Caspr/paranodin (green). Caspr2 and Kv1.1 labeling were dramatically altered in mutant mice, whereas the other markers appeared normal. Protein 4.1B-IR (red) was intense in the paranodal regions (arrows) where it was colocalized with Caspr/paranodin (green), and was also visible in the juxtaparanodal regions (arrowheads) of wild-type and mutant mice. Bars: (A–J) 20 μ m and (K and L) 5 μ m. (M and N) Distribution of Caspr2, TAG-1 and Kv1.2 in teased fibers of sciatic nerve of a P8 rat. (M) Caspr2 (red) and (N) TAG-1 (red) were detected in a limited number of fibers, where they appeared colocalized with (M and N) Kv1.2 potassium channels (green). Bar, 2.5 μ m.

paranodin/Caspr (Fig. 4 A), as reported previously (Traka et al., 2002). In mutant mice, TAG-1-IR was absent, as expected, whereas paranodin/Caspr-IR appeared normal (Fig. 4 B). The glial protein NF155 was also properly localized at paranodes (Fig. 4, C and D) and sodium channels were normally restricted to the nodes in nerves of mice of both genotypes (Fig. 4, E and F). Myelin-associated glycoprotein (MAG) was normally enriched in paranodal regions, as well as at Schmidt-Lanterman incisures in wild-type and TAG-1 mutant mice (Fig. 4, G and H). Thus, in the absence of TAG-1, the major nodal and paranodal proteins were normally localized in sciatic nerves.

In contrast, the molecular organization of the juxtaparanodal regions of the TAG-1 mutant sciatic nerves was severely altered. We were unable to detect localized Caspr2 labeling in the mutants compared with wild-type mice (Fig. 4, G and H), and a dramatic reduction of the Kv1.1-IR was observed in TAG-1-deficient mice (Fig. 4, I and J). Similar results were obtained for Kv1.2 (unpublished data). Quantitative analysis of Kv1.1 and Kv1.2 channel distribution in mutant sciatic nerve fibers showed all the nodal sites to be affected: 417 nodes from four mice were analyzed for Kv1.1, and 233 nodes from two mice for Kv1.2; K⁺ channels accumulation was undetectable in 70% of the mutant juxtaparanodes examined, whereas a reduced clustering, as in Fig. 4 J, was observed in the rest of the sites. IB analysis showed that Caspr2 protein levels were unchanged in TAG-1 mutant sciatic nerves (106 \pm 22% of wild type, mean \pm SD, n = 3), whereas the levels of the immature band of Kv1.1 was increased (194 \pm 40% of wild-type levels, mean \pm SD, n = 3). Thus, the dramatic alteration of

Caspr2 and K⁺ channels enrichment in the absence of TAG-1, demonstrated their failure to accumulate in juxtaparanodal regions of peripheral myelinated fibers and not an expression defect.

Protein 4.1B is enriched at paranodes and, to a lesser degree, at juxtaparanodes (Ohara et al., 2000), where it interacts with the intracellular domain of Caspr/paranodin and Caspr2, respectively (Gollan et al., 2002; Denisenko-Nehrbass et al., 2003b). We examined the localization of protein 4.1B in TAG-1 mutants (Fig. 4, K and L). In wild-type mice protein 4.1B-IR was highly concentrated at paranodes (Fig. 4 K, arrows) and was also present at juxtaparanodes (Fig. 4 K, arrowheads). In TAG-1-deficient mice the localization of protein 4.1B at paranodes was unchanged, whereas only a small decrease was observed at juxtaparanodes (Fig. 4 L). Quantification in three wild-type and four mutant nerves revealed that the proportion of juxtaparanodes where 4.1B-IR was clearly visible, was 92 \pm 1% and 70 \pm 3%, respectively (mean \pm SEM, P < 0.01, t test). Altogether these observations demonstrated that in the absence of TAG-1 the juxtaparanodal enrichment of Caspr2 was lost and that of K⁺ channels was severely disrupted. In contrast, protein 4.1B was only moderately affected, indicating that its juxtaparanodal localization is largely independent of the presence of Caspr2.

TAG-1, Caspr2, and K⁺ channels are colocalized early during myelination

Because our data indicated a role of TAG-1 in the targeting of Caspr2 and K⁺ channels, it was important to determine whether these three proteins were found at the same

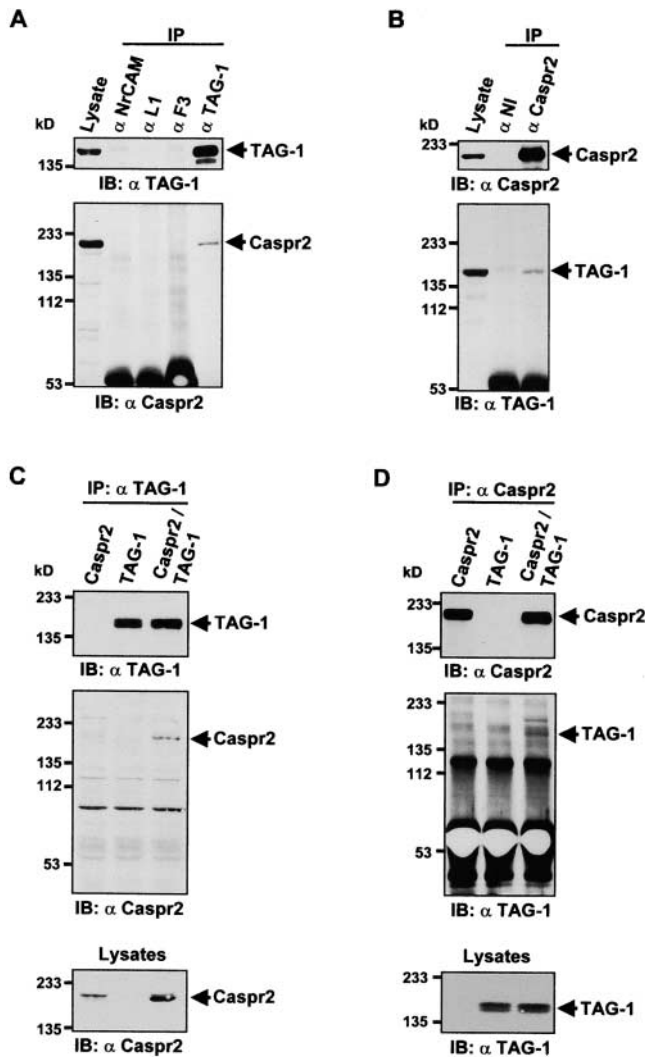


Figure 5. Association of TAG-1 and Caspr2 in brain and transfected COS-7 cells. (A and B) Association of TAG-1 and Caspr2 in brain. Rat brain proteins were extracted and subjected to IP with (A) α Caspr2 or (B) α TAG-1. The presence of specific proteins in the precipitates was examined by IB with the indicated antibodies. Aliquots of crude protein extracts (Lysate, 1/60 of protein amount used for each colP) were also subjected to IB to verify the expression of the proteins. (A) Caspr2 was detected in immune precipitates with TAG-1 antibodies but not with antibodies against other IgSF proteins (α NrCAM, α L1, and α F3). (B) TAG-1 was detected in immune precipitates with Caspr2 antibodies but not nonimmune serum (α NI). (C and D) Association of TAG-1 and Caspr2 in transfected COS-7 cells. Lysates from COS-7 cells overexpressing either Caspr2 or TAG-1 alone, or both, were prepared as described in Materials and methods and subjected to IP either with (C) α TAG-1 or (D) α Caspr2. Precipitates were resolved by SDS-PAGE, analyzed by IBs with antibodies against Caspr2 and TAG-1, to detect the presence of specific proteins (C and D, top and middle). Aliquots of crude protein extracts (Lysates) were also subjected to immunoblotting to verify the expression of the proteins (C and D, lower panels). TAG-1 antibodies colP Caspr2 only in doubly transfected cells (C), whereas Caspr2 antibodies pulled-down TAG-1 only in cotransfected cells (D). Note the slight shift of migration of Caspr2 in the presence of TAG-1, which results in a doublet with a predominant lower band in cotransfected cells (C, Lysates). The position of molecular mass markers (kD) is indicated.

locations early during development. We examined the localization of TAG-1, Caspr2, and Kv1.2 in rat sciatic nerve at postnatal day 8 (P8), a time around which K^+ channels appear in a few fibers, transiently localized at nodes and paranodes, and then progressively to the juxtaparanodes (Vabnick et al., 1999), whereas Caspr2 has been reported to follow K^+ channel distribution (Poliak et al., 2001). At P8, localized enrichment of these proteins was detected in a limited number of fibers (Fig. 4, M and N). We confirmed the colocalization of Caspr2 and Kv1.2 (Fig. 4 M), and we found that TAG-1-IR always overlapped with Kv1.2-IR (Fig. 4 N). These results indicate that TAG-1 is colocalized with Caspr2 and Kv1.2 channels early during development, and support its involvement in the targeting of these proteins.

TAG-1 and Caspr2 are associated in brain and in transfected cells

The colocalization of TAG-1 and Caspr2 in mice and rats, together with the mislocalization of Caspr2 in TAG-1-deficient mice prompted us to examine the possibility that these proteins form a complex at juxtaparanodes by performing coimmunoprecipitation experiments from brain extracts. Caspr2 was detected in TAG-1 immune precipitates but not in immunoprecipitation (IP) performed with antibodies against other IgSF proteins (Fig. 5 A). Conversely, TAG-1 was specifically detected in Caspr2 immune precipitates (Fig. 5 B). These results indicate the existence of a specific association between TAG-1 and Caspr2 *in vivo*. We examined further the association between TAG-1 and Caspr2 using COS-7 cells transfected with expression plasmids for either of these proteins, alone or in combination (Fig. 5, C and D). IP with TAG-1 antibodies pulled down Caspr2 in cells doubly transfected with TAG-1 and Caspr2 but not in cells expressing only Caspr2 (Fig. 5 C). On the other hand, Caspr2 antibodies coprecipitated TAG-1 only in doubly transfected cells (Fig. 5 D).

TAG-1 and Caspr2 form a cis complex at the plasma membrane

We investigated whether the association of TAG-1 and Caspr2 altered their subcellular localization in transfected COS-7 cells. When transfected alone, Caspr2 or TAG-1 were, for the most part, localized at the level of the plasma membrane (Fig. 6, A and B). However, the IR pattern of these two proteins was different because Caspr2 appeared rather uniformly distributed, whereas TAG-1-IR had a punctate or patchy appearance, as reported previously in transfected CHO cells (Buttiglione et al., 1998). When both proteins were simultaneously expressed, they were largely colocalized at the plasma membrane, and their distribution appeared similar to that of Caspr2 alone (Fig. 6 C).

TAG-1 is a GPI-anchored protein found in cholesterol-rich, Triton X-100-insoluble membrane fractions (Buttiglione et al., 1998; Kasahara et al., 2000; Prinetti et al., 2001). In transfected COS-7 cells, TAG-1 was Triton X-100 insoluble at 4°C, whereas Caspr2, a type 1 transmembrane protein, was soluble (unpublished data). We examined the

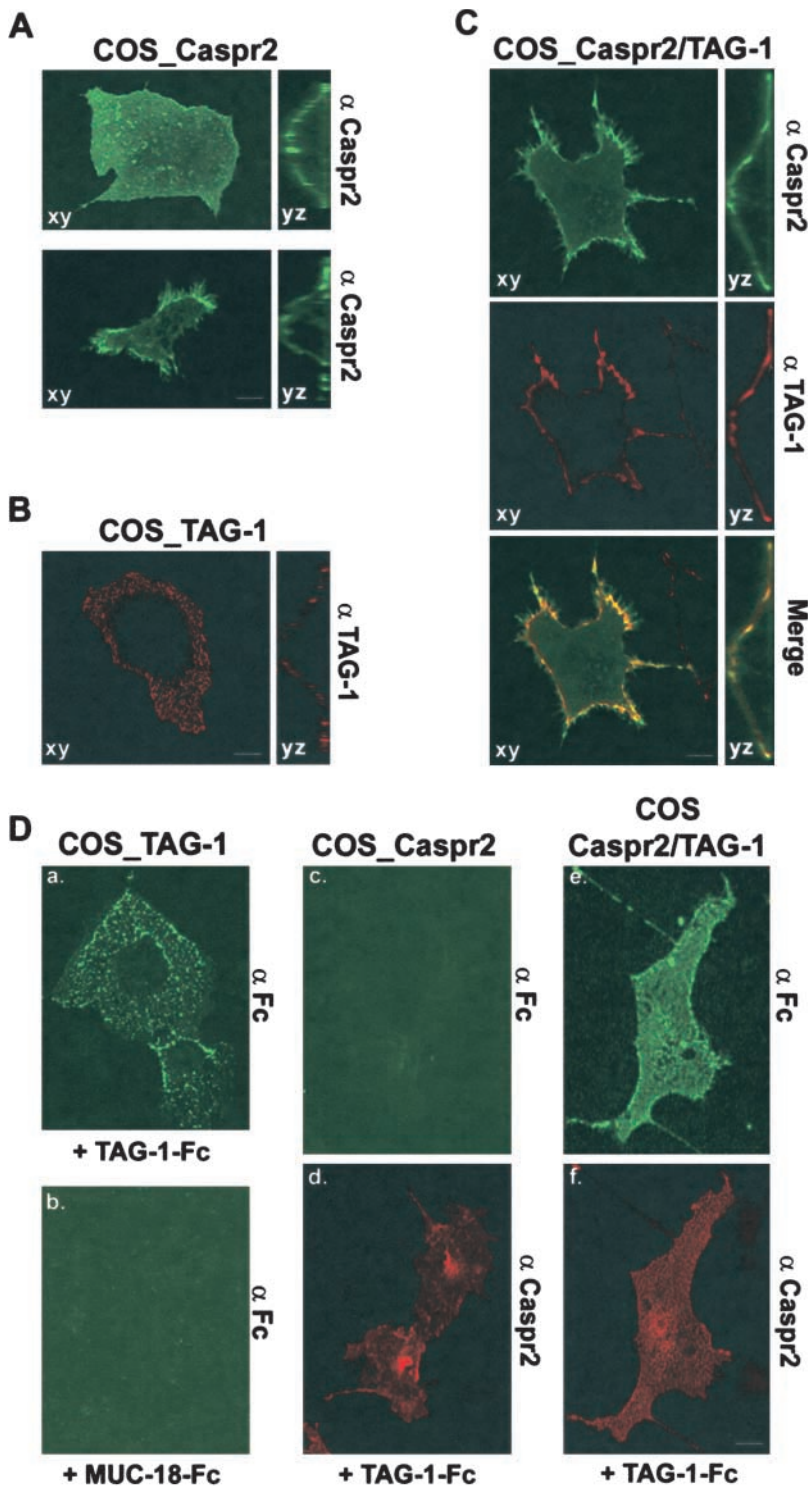


Figure 6. Cis and trans interactions between TAG-1 and Caspr2 in COS-7 cells. (A–C) Colocalization of Caspr2 and TAG-1 at the plasma membrane of COS-7 cells. COS-7 cells overexpressing either Caspr2 or TAG-1 alone, or both, were processed for indirect IF and laser confocal microscopy analysis. Caspr2 was uniformly localized at the plasma membrane (A, COS_Caspr2). TAG-1 displayed a membrane localization with a patchy appearance (B, COS_TAG-1). In cotransfected cells, Caspr2 (green) and TAG-1 (red) were largely colocalized (yellow) at the plasma membrane with a distribution similar to that of Caspr2 alone (C, COS_Caspr2/TAG-1). Single confocal sections are shown. (D) Association of TAG-1 in trans with itself but not with Caspr2. COS-7 cells overexpressing either TAG-1 alone (COS_TAG-1, a and b), Caspr2 (COS_Caspr2, c and d) or both (COS_Caspr2/TAG-1, e and f) were incubated with a (a and c–f) TAG-1-Fc chimeric protein or a (b) MUC-18-Fc chimera protein, and processed for indirect IF and laser confocal microscopy analysis. The TAG-1-Fc chimeric protein (green) bound to cells expressing (a) TAG-1 alone or in combination with (e) Caspr2 but not to cells expressing (c) Caspr2 alone. Expression of Caspr2 was verified by (d and f) specific IF (red). Stacked confocal images of eight consecutive sections 1 μm apart. Bar, (A–D) 10 μm .

membrane compartments in which Caspr2 and TAG-1 were distributed and whether their coexpression altered this distribution by loading Triton X-100 cell lysates on discontinuous sucrose gradients (Fig. 7). Analysis of the proteins in the fractions revealed that TAG-1 was present in the 10 and 25% sucrose fractions (Fig. 7 A, top). In contrast, Caspr2 expressed alone was recovered in the heavier 40% fraction and in the pellet (Fig. 7 B, top). When coexpressed with Caspr2, TAG-1 was no longer present in the light fractions

but was only found in the pellet with Caspr2 (Fig. 7, A and B, bottom). These experiments indicate that when Caspr2 and TAG-1 are coexpressed, their interaction alters the distribution of TAG-1 by changing its microenvironment and/or by linking the complexes to cytoplasmic components. Altogether our results reveal that Caspr2 is able to reach the plasma membrane by itself, and that Caspr2 and TAG-1 are colocalized and associated in cis at the plasma membrane in transfected cells.

TAG-1 associates in trans with itself but not with Caspr2

The results reported above underline the ability of TAG-1 to associate with Caspr2 in the same membrane. However, TAG-1 is also highly expressed in myelinating glial cells and its enrichment in juxtaparanodes is likely to result, at least in part, from its accumulation in the glial membrane (Traka et al., 2002). This localization suggested that TAG-1 could interact with the TAG-1–Caspr2 complex in the facing axolemma. To examine the possibility of such trans interactions, we incubated COS-7 cells expressing TAG-1 and/or Caspr2 with a TAG-1-Fc chimeric protein (Fig. 6 D). TAG-1-Fc was readily capable to interact with cells transfected with TAG-1 (Fig. 6 D), confirming its well-characterized homophilic recognition properties (Tsiotra et al., 1996; Pavlou et al., 2002). The specificity of the interaction was indicated by the lack of binding of a MUC-18-Fc chimera to these cells (Fig. 6 D, b). The TAG-1-Fc chimeric protein did not bind to cells transfected with Caspr2 alone (Fig. 6 D, c and d), whereas it interacted with cells cotransfected with Caspr2 and TAG-1 (Fig. 6 D, e and f). The binding of TAG-1-Fc to cells expressing only TAG-1 or TAG-1 and Caspr2 appeared similar. These results show that TAG-1 was not able to interact in trans with Caspr2, but that the presence of Caspr2 did not alter the capacity of TAG-1 to establish trans homophilic interactions.

Discussion

Axo–glial interactions result in a highly segregated distribution of membrane proteins, defining distinct domains of the axolemma. The mechanisms leading to the enrichment of Na⁺ channels and associated proteins at the nodes of Ranvier, as well as those involved in the formation of paranodal axo–glial junctions, have been extensively investigated (for review see Girault and Peles, 2002). In contrast, hardly anything is known about the basis for the accumulation of specific proteins, including potassium channels, at juxtaparanodes. The present work demonstrates the critical role of TAG-1 for the enrichment of axonal proteins Caspr2 and Kv1.1/Kv1.2 in juxtaparanodal regions, and points out unexpected molecular similarities in axo–glial interactions at paranodes and juxtaparanodes.

Despite the lack of major ultrastructural or functional alterations of myelinated fibers in TAG-1–deficient mice, a detailed analysis revealed that the normal distribution of the known molecular components of the juxtaparanodal region was selectively disturbed in the CNS and PNS of these animals. Although the localization of Na⁺ channels and paranodal proteins was normal in TAG-1 mutant mice, the normal accumulation of Caspr2 at juxtaparanodes was completely lost and the distribution of delayed rectifier K⁺ channels was severely altered. Thus, the phenotype of TAG-1–deficient mice is markedly different from that of other mutant strains described so far. For example, deletion of oligodendrocytes in transgenic mice during the first days after birth induces a virtually complete absence of organization of axonal proteins beyond the initial segments (Mathis et al., 2001). Dysmyelination in *jimpy* mice or *md* rats, as well as targeted mutations in the galactolipid biosynthetic pathway, severely alter the or-

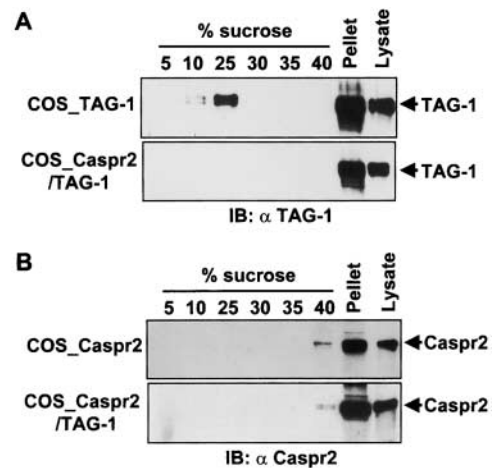


Figure 7. Sucrose density gradient of TAG-1 and Caspr2 in transfected COS-7 cells. COS-7 cells overexpressing either (A, top, COS_TAG-1) TAG-1 alone or (B, top, COS_Caspr2) Caspr2 alone, or (A and B, bottom, COS_Caspr2/TAG-1) both proteins, were lysed in a Triton X-100 containing buffer and lysates were submitted to discontinuous sucrose gradients as described in Materials and methods and IB with antibodies against (A) TAG-1 and (B) Caspr2. Aliquots of crude protein extracts (Lysate) were also analyzed to verify the expression of the proteins. TAG-1 expressed alone was present in the 10 and 25% sucrose fractions (A, top). In contrast, Caspr2 was recovered in the heavier 40% fraction and in the pellet (B, bottom). When coexpressed with TAG-1, TAG-1 was no longer present in the light fractions but was found in the pellet with Caspr2 (A and B, bottom).

ganization of paranodal junctions without preventing the initial accumulation of K⁺ channels in direct contact with the nodes (Dupree et al., 1999; Mathis et al., 2001; Arroyo et al., 2002). Targeted mutations of paranodal proteins prevent the formation of septate-like junctions and also result in a lack of separation between K⁺ channels and Na⁺ channels clusters (Bhat et al., 2001; Boyle et al., 2001). These observations support a role of fence for the paranodal junction (Pedraza et al., 2001), separating the internode from the node. They also strongly indicate that the mechanisms leading to the accumulation of K⁺ channels and Caspr2 in the juxtaparanodal regions are relatively independent from those governing the formation of nodes and paranodal junctions. Thus, the phenotype of TAG-1 mutants provides novel insights into the organization of axonal domains.

Our results show that TAG-1 is closely associated with Caspr2 and is required for its accumulation at juxtaparanodes by recruiting and/or stabilizing it at this location. Co-IP experiments demonstrated that Caspr2 and TAG-1 form a complex in brain and in transfected cells. In addition, the two proteins were colocalized at the plasma membrane, and the presence of Caspr2 modified TAG-1 membrane distribution, which became more diffuse in intact cells and disappeared from the light fractions in sucrose gradients. These results indicate that the association of the two proteins alters significantly their membrane microenvironment and/or their interaction with other proteins.

Our findings in COS-7 cells demonstrate that TAG-1 can exchange cis interactions with Caspr2. This ability supports an association between the two proteins in the axolemma be-

cause TAG-1 is expressed in several types of neurons (Dodd et al., 1988; Karagozeos et al., 1991), including adult neurons of the dorsal root ganglia and their projections (unpublished data) and spinal motor neurons (Traka et al., 2002). However, TAG-1 is also expressed in Schwann cells and oligodendrocytes and could be expected to exchange trans interactions with Caspr2. We tested this possibility using TAG-1-Fc chimeras and did not observe any binding, suggesting that in these conditions the two proteins interact directly only if they are present in the same membrane, in the same orientation. Yet, in these assays, TAG-1-Fc was readily capable to bind to membrane-bound TAG-1, in the absence or presence of cotransfected Caspr2. Thus, our results are compatible with a model in which TAG-1 interacts in cis with Caspr2 in the axolemma and in trans, through homophilic interaction, with another molecule of TAG-1 in the glial membrane (Fig. 8). A precedent for this type of interaction has been shown to occur between TAG-1 and L1 (Malhotra et al., 1998). In that case, the trans homophilic interaction between TAG-1 molecules resulted in cis activation of L1, inducing its binding to ankyrin. Although the model depicted in Fig. 8 is the simplest that accounts for all the presently available data, the possibility that additional components are part of this macromolecular complex cannot be excluded, as TAG-1 has been shown to interact with several other extracellular proteins (Milev et al., 1996; Pavlou et al., 2002). In addition, the association of Caspr2 with protein 4.1B (Denisenko-Nehrbass et al., 2003b) suggests that the TAG-1–Caspr2 ternary complexes may be attached to the cytoskeleton through this protein that has the capability to interact with actin and spectrin (Gimm et al., 2002). In TAG-1 knockout mice, we found that protein 4.1B was still present in juxtapanodal regions although Caspr2-IR was not accumulated in these regions. This observation indicates that additional targeting mechanisms account for the localization of protein 4.1B to juxtapanodes, and that the presence of protein 4.1B is not sufficient to induce the accumulation of Caspr2 in these regions. Therefore, we suggest that the combination of two complementary mechanisms may be required for the normal localization of the TAG-1–Caspr2 ternary complexes at juxtapanodes: an axo–glial, TAG-1–mediated, homophilic interaction, and the anchoring of Caspr2 to cytoskeletal elements in the axon that may be found only in the vicinity of nodes of Ranvier. A prediction of this model is that TAG-1 localization should be altered in the absence of Caspr2 or of protein 4.1B.

An important conclusion of this paper is that TAG-1 is also essential for K^+ channels enrichment at juxtapanodes because a severely disrupted distribution of Kv1.1 and Kv1.2 was observed in mutant mice. It should be pointed out that the normal basic electrophysiological properties of TAG-1–deficient sciatic nerves is not surprising given the known insensitivity of adult sciatic nerves to K^+ channel blockers (Vabnick et al., 1999) and the absence of dysmyelination in TAG-1 mutants (this paper). Further analysis of TAG-1–deficient mice might be useful to address the function of the juxtapanodal K^+ channels. At any rate, this paper provides insights into the mechanisms of enrichment of these channels. These channels can coprecipitate with Caspr2 and there is indirect evidence that PDZ domain proteins are nec-

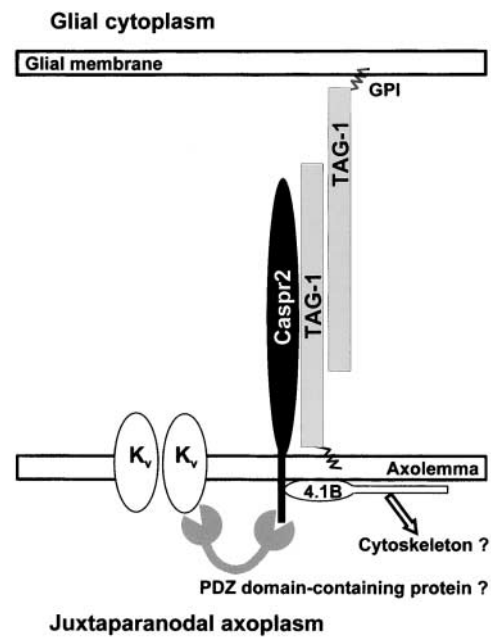


Figure 8. Model of the molecular organization of juxtapanodal regions. This model is the simplest that can account for the data from previous studies and the present work. Caspr2 is enriched in the axolemma, whereas TAG-1 is expressed in both neurons and myelinating glial cells. Experiments in transfected cells show that TAG-1 interacts with Caspr2 in cis, and that TAG-1 exchanges trans interactions with itself (homophilic) but not with Caspr2. The functional importance of these interactions is demonstrated by the absence of Caspr2 enrichment in juxtapanodal regions in TAG-1 knockout mice. These complexes are associated with other proteins including Kv1.1 and Kv1.2 potassium channels, presumably through a PDZ domain–containing protein (schematically represented here; Poliak et al., 1999; Rasband et al., 2002) and with protein 4.1B (Denisenko-Nehrbass et al., 2003b). Because protein 4.1B was still detected at juxtapanodes in the absence of TAG-1 and Caspr2, it is likely that it interacts with other proteins, including components of the cytoskeleton (arrow). Correct localization of juxtapanodal proteins may depend on both axo–glial interactions and binding to axoplasmic cytoskeletal components.

essary for this association (Poliak et al., 1999; Rasband et al., 2002; Fig. 8). Alternatively, the accumulation of K^+ channels could originate from their interaction with TAG-1, a possibility that remains to be formally ruled out. Interestingly, some K^+ channel accumulation was still observed in the vicinity of paranodes of 2-mo-old TAG-1–deficient mice in the absence of detectable Caspr2, indicating the minor contribution of additional targeting mechanism(s).

Paranodin/Caspr and contactin/F3, are essential for the formation of septate junctions where their glial partner is NF155, a transmembrane IgSF. Moreover, neurexin IV, the *Drosophila* member of the NCP family is essential for septate junction formation in flies (Baumgartner et al., 1996) and may form a tripartite complex with D-contactin and neuroglian, the orthologues of contactin/F3 and NF155, respectively (Faivre-Sarrailh, C., and M. Bhat, personal communication). Therefore, a core tripartite complex encompassing a NCP and a contactin-like protein in one membrane and an IgSF protein in the facing membrane appears to be a conserved molecular building block of intercellular contacts.

The latter IgSF protein can be a transmembrane protein in the case of paranodal septate-like junctions and *Drosophila* septate junctions, or a contactin-like molecule in the case of juxtaparanodes (this paper).

The molecular similarities between paranodal and juxtaparanodal protein complexes contrast with the striking ultrastructural differences between these two regions. At paranodes, the plasma membranes are separated by a narrow gap, interrupted by regularly spaced electron dense material that forms septa, in register with regularly organized intramembrane particles in glial and axonal membranes, as detected by freeze fracture (Wiley and Ellisman, 1980). In contrast, at juxtaparanodes the membranes are more loosely apposed and do not display septate-like junctions. Freeze fracture has only revealed the presence of sparse particles in juxtaparanodal axonal and glial membranes (Stolinski et al., 1981; Tao-Cheng and Rosenbluth, 1984), which may correspond to ion channels and, possibly, to Cx 29 hemichannels (Stolinski et al., 1981; Tao-Cheng and Rosenbluth, 1984; Li et al., 2002). Thus, the conserved NCP-IgSF ternary complexes appear to be involved in strikingly distinct types of cell-cell contacts. A noticeable difference is that NF155, the glial moiety of this complex at paranodes, is a transmembrane protein, presumably associated with the cytoskeleton, as in *Drosophila* septate junctions, whereas at juxtaparanodes, TAG-1 is a GPI-anchored protein. Further experiments will be required to determine whether this difference accounts for the striking differences between paranodal and juxtaparanodal NCP-contactin-based intercellular complexes, or whether additional components are involved. In either case, the present work demonstrates the contribution of TAG-1 and associated Caspr2 in the organization of axonal domains at nodes of Ranvier.

Materials and methods

Mice

TAG-1 knockout mice were produced as described previously (Fukamauchi et al., 2001) and kept as heterozygote breeding pairs. The housing and animal procedures used were in agreement with European Union policy.

Standard EM

Two wild-type mice and two mutant littermates were deeply anesthetized by Avertin (0.425 mg/g; i.p.), perfused transcardially with 2% PFA/2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 1 mM CaCl₂. The spinal cords were immersed in the fixative for 2 h at RT. Samples were rinsed in 0.1 M cacodylate buffer containing 4% sucrose and fixed after in 2% OsO₄, rinsed, and dehydrated in an ascending series of ethanol. The samples were embedded flat in a mixture of Epon and Araldite. Ultra-thin sections were stained with uranyl acetate and lead citrate and were examined with an electron microscope (model EM 201C; Philips).

Electrophysiological studies

Studies were performed on sciatic nerves of mice anesthetized with Avertin (0.5 mg/g; i.p.) with temperature maintained at 31°C. Recording needle electrodes were placed subcutaneously in the footpad. Supramaximal stimulation of sciatic nerves was performed with a 0.1–0.2 ms rectangular pulse, stimulating distally at the ankle and proximally at the sciatic notch with needle electrodes. Recordings were obtained on a TECA Neurostar (Oxford Instruments) with a filter setting of 2 Hz to 10 kHz. Latencies correspond to the time lapse between the stimulus and the onset of CMAPs. Conduction velocities were calculated as follows: conduction velocities = distance / (proximal latency – distal latency). The peak to peak amplitudes of CMAPs were measured and the ratio of proximal versus distal amplitude was used to determine the presence or absence of partial conduction block.

Antibodies and constructs

The mAb 1C12 against TAG-1 was used for IP and IF of transfected cells, whereas polyclonal antibodies for immunostaining of teased fibers and IBs were used (Dodd et al., 1988; Traka et al., 2002). Polyclonal antisera against paranodin/Caspr (L-51) and Caspr2 have been described previously (Menegoz et al., 1997; Denisenko-Nehrbass et al., 2003b). mAbs against MAG (Poltorak et al., 1987), and Caspr (Poliak et al., 1999), and the polyclonal antibodies against NF155 (Tait et al., 2000), F3, NrCAM, and L1 were provided by M. Schachner (Zentrum für Molekulare Neurobiologie, Hamburg, Germany), E. Peles (The Weizmann Institute, Rehovot, Israel), P. Brophy (University of Edinburgh, Edinburgh, UK), C. Faivre-Saraille (Institute of Jean Roche, Marseille, France), and T. Galli (UMPC, Paris, France). The sodium channel (PAN) mAb was purchased from Sigma-Aldrich; Kv1.1 and Kv1.2 mAbs were purchased from Upstate Biotechnology; actin mAb was purchased from Amersham Biosciences; polyclonal anti-human Fc, goat anti-rabbit Cy3, and goat anti-mouse Cy3 antibodies were purchased from Jackson ImmunoResearch Laboratories; and goat anti-rabbit Alexa Fluor 488, anti-mouse Alexa Fluor 488, and anti-mouse Alexa Fluor 594 antibodies were purchased from Molecular Probes. HRP-conjugated goat anti-mouse and anti-rabbit antibodies used for immunoblotting were purchased from Amersham Biosciences.

For expression of TAG-1 in COS-7 cells, Pc-TAG consisting of the entire coding region of rat TAG-1 cloned in the pcDNA1 vector (Invitrogen; Buttiglione et al., 1998) was used. The human Caspr2 cDNA (NM_014141, KIAA0868), provided by O. Ohara (Kazusa DNA Research Institute, Kisarazu, Japan), was introduced into the KpnI/NotI sites of the pcDNA3 vector (Invitrogen). Production of TAG-1-Fc (human homologue of rat TAG-1 fused to the Fc region of the human IgG1) and MUC-18-Fc was as described previously (Buttiglione et al., 1998; Pavlou et al., 2002). In the transient transfection experiments to detect Fc binding, the human homologue of TAG-1 cloned in the pcDNA3 vector was used (Pavlou et al., 2002).

Immunohistofluorescence

Sciatic and optic nerves from adult wild-type and mutant animals, as well as sciatic nerves from P8 rats, were dissected and fixed in 2% PFA for 30 min at RT. Sciatic nerves were teased apart to yield single fiber preparations, air dried overnight at RT, and kept at –80°C. For immunostaining, teased nerve fibers, and optic nerve, 10–12 μm frozen sections were used. All tissue samples were incubated in –20°C acetone for 10 min. Specimens were further processed as described in Traka et al. (2002). Images were acquired by a laser scanning microscope (model SP; Leica) using a 40 or 63× oil objective.

Biochemical experiments

Specific protein levels in sciatic and optic nerves were performed as described previously and normalized to actin levels (Traka et al., 2002). IPs from 6 mg protein/IP of brain extracts and 1 mg/IP of transfected COS-7 cells were performed essentially as described previously (Denisenko-Nehrbass et al., 2003a), except that the extraction buffer contained 85 mM Tris, pH 7.5, 30 mM NaCl, 1 mM EDTA, 120 mM glucose, 1% Triton X-100, 60 mM *n*-octylglucoside, and 1 mM PMSF. Isolation of low density Triton X-100-insoluble complexes from transfected COS-7 cells was performed as described previously (Buttiglione et al., 1998) except that the sucrose gradient was discontinuous. Proteins of interest were detected by IB and ECL chemiluminescent detection (Amersham Biosciences).

Cell culture, IF, and Fc-binding procedures

COS-7 cells were transfected using Polyethylenimine or Fugene 6 (Roche) using 8 μg of plasmid/10-mm-diam dish and 1–2 μg of plasmid/35-mm-diam dish. After transfection, cells were grown for 24 h before processing. For TAG-1 detection, indirect labeling was performed on living cells washed once with PBS, incubated with mAb 1C12 diluted in PBS/1% BSA for 30 min at RT, washed twice with PBS, and incubated with the goat anti-mouse Alexa Fluor 594 for 30 min at RT. For Caspr2 detection, cells immunostained for TAG-1 were fixed for 20 min in 4% PFA, permeabilized with 0.02% Triton X-100 for 5 min, and incubated with the anti-Caspr2 antibody and goat anti-rabbit Alexa Fluor 488 for 30 min each at RT. Coverslips were mounted in Vectashield. For binding assays on TAG-1 or Caspr2 transfected COS-7 cells, TAG-1-Fc and MUC-18-Fc Fc-chimeras (15 μg/ml of each) were cross-linked with anti-human Fc antibody (50 μg/ml) for 1 h at 37°C and incubated as described previously (Buttiglione et al., 1998; Pavlou et al., 2002). Detection of the binding was achieved after fixation and permeabilization with anti-rabbit Alexa Fluor 488 antibody. Caspr2 was detected with polyclonal antibody and anti-rabbit Cy3 antibody. Cells were mounted in Mowiol (Calbiochem) and the images were acquired using a laser scanning microscope (model SP; Leica).

We are grateful to Drs. Brophy, Ohara, Peles, Schachner, Faivre-Sarrailh, and Galli for providing reagents and Dr. Isidoridou (Neurobiology Research Institute, Cozzika Foundation) for the EM facility; to Dr. Brian Popko for constructive comments throughout the course of this work; and to Drs. Popko and Strigini for comments on the manuscript. We thank M. Mouratidou, M. Carnaud, and K. Oguievetskaia for their help at the final stages of this work and N. Campbell for her excellent technical assistance.

This work was supported by grants from the National Society for Multiple Sclerosis [RG3368], the IMBB, the Onassis Foundation, and the Greek Ministry of Education [EPEAEK 1092] (to D. Karagozeos); Fondation Schlumberger pour l'Education et la Recherche, Association Française contre les Myopathies, and Fondation pour la Recherche Médicale (to J.A. Girault); and National Institutes of Health RO1 NS39346-01, a gift from M.P. Miller through the Brain Research Foundation, and the Jack Miller Neuropathy Center (to B. Soliven).

Submitted: 15 May 2003

Accepted: 28 July 2003

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