A role for Sec8 in oligodendrocyte morphological differentiation

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Summary

In the central nervous system, oligodendrocytes synthesize vast amounts of myelin, a multilamellar membrane wrapped around axons that dramatically enhances nerve transmission. A complex apparatus appears to coordinate trafficking of proteins and lipids during myelin synthesis, but the molecular interactions involved are not well understood. We demonstrate that oligodendrocytes express several key molecules necessary for the targeting of transport vesicles to areas of rapid membrane growth, including the exocyst components Sec8 and Sec6 and the multidomain scaffolding proteins CASK and Mint1. Sec8 overexpression significantly promotes oligodendrocyte morphological differentiation and myelin-like membrane formation in vitro; conversely, siRNA-mediated interference with Sec8 expression inhibits this process, and anti-Sec8 antibody induces a reduction in oligodendrocyte areas. In addition, Sec8 colocalizes, coimmunoprecipitates and cofractionates with the major myelin protein OSP/Claudin11 and with CASK in oligodendrocytes. These results suggest that Sec8 plays a central role in oligodendrocyte membrane formation by regulating the recruitment of vesicles that transport myelin proteins such as OSP/Claudin11 to sites of membrane growth.

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Introduction

Myelin is a highly polarized, metabolically active membrane that promotes saltatory conduction and participates in bi-directional signaling between neurons and oligodendrocytes (OLs) (Pfeiffer et al., 1993; Barres and Raff, 1999; Menon et al., 2003). Myelin membrane biogenesis, a defining process for OLs, requires coordinated sorting and recruitment of specialized proteins, lipids and mRNAs to distinct membrane subdomains (Trapp et al., 1995; Arroyo and Scherrer, 2000; Pedraza et al., 2001; Salzer, 2003; Trapp et al., 2004). This process has been investigated by analogy to polarized transport in epithelial cells (Minuk and Braun, 1996; deVries et al., 1998; deVries et al., 2000; Kroepfl and Gardinier, 2001) and neuronal synapses, leading to the identification of two classes of transport proteins, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) and Rab-GTPases (Huber et al., 1994; Madison et al., 1996; Burcelin et al., 1997; Madison et al., 1999; Rodriguez-Gabin et al., 2001; Rodriguez-Gabin et al., 2004). Of these, the v-SNARE synaptobrevin-2 and Rab3a-GTPase are upregulated in mature OLs, suggesting that they play specific roles in myelination (Huber et al., 1994; Madison et al., 1999).

In addition to SNAREs and Rab-GTPases, other classes of molecules play essential roles in membrane formation and polarization, including the exocyst complex and multidomain scaffolding proteins (Roh and Margolis, 2003; Nelson, 2003). The exocyst is an octameric complex (Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84) involved in the recruitment of transport vesicles to areas of rapid membrane growth, a process followed by SNARE-mediated vesicle fusion (Novick et al., 1980; Ting et al., 1995; TerBush et al., 1996; Grindstaff et al., 1998; Hazuka et al., 1999). The exocyst is also important during earlier steps in the transport pathway, specifically in post-translational regulation of protein synthesis (Lipschutz et al., 2000; Lipschutz et al., 2003). These functions are essential, because an inactivating mutation in the Sec8 gene is embryonic lethal in mice, and Sec5, Sec6 and Sec10 are necessary for survival in *Drosophila melanogaster* (Friedrich et al., 1997; Andrews et al., 2002; Murthy et al., 2003; Murthy et al., 2005; Beronja et al., 2005; Mehta et al., 2005). In epithelial cells, through interactions with adhesion molecules, exocyst components are recruited to intercellular junctions where they control protein recruitment to basolateral membranes (Grindstaff et al., 1998; Yeaman et al., 2001; Yeaman et al., 2004). In neurons, members of the exocyst are involved in neurite outgrowth, and together with multidomain scaffolding proteins control receptor transport to the synapse (Hsu et al., 1998; Hazuka et al., 1999; Vega and Hsu, 2001; Sans et al., 2003). In mammalian cells, small GTPases such as RalA, TC10, Arf-6 or Rab11 regulate exocyst membrane recruitment and function (Brymora et al., 2001; Moskalenko et al., 2002; Sugihara et al., 2002; Inoue et al., 2003; Prigent et al., 2003; Zhang et al., 2004). Although the mechanism is still unclear, it has been proposed that exocyst proteins form subcomplexes (to which different components are sequentially added and/or removed during trafficking to the plasma membranes), and that

various members of the complex may be functionally heterogeneous (Moskalenko et al., 2003; Vik-Mo et al., 2003; Murthy et al., 2003; Murthy and Schwartz, 2004; Beronja et al., 2005).

Another level of exocyst functional regulation is provided by interactions between its components (e.g. Sec8) and multidomain scaffolding proteins, such as membrane-associated guanylate kinases (MAGUKs) (Sans et al., 2003; Yeaman et al., 2004). These are large molecules that contain multiple protein interaction domains and can form regulatory complexes involved in vectorial transport and membrane polarization (Hata et al., 1996; Gundelfinger and tom Dieck, 2000; Setou et al., 2000; Zhang et al., 2001; Lee et al., 2002; Leonoudakis et al., 2004). For example, CASK (calcium/calmodulin-dependent serine kinase), a member of the MAGUK family, associates with Mint1 and Velis in a complex involved in protein recruitment to neuronal synaptic junctions and to epithelial basolateral membranes (Butz et al., 1998; Setou et al., 2000; Martinez-Estrada et al., 2001; Lee et al., 2002; Olsen et al., 2002; Leonoudakis et al., 2004).

In the present study we show that Sec8, a representative exocyst component, plays key roles in regulating OL morphological differentiation and membrane formation, and we identify two new Sec8 binding partners, CASK and OL-specific protein (OSP/Claudin11), a major component of the myelin membrane.

Results

Sec8 and Sec6 are present in OLs

We studied the expression of Sec8 and Sec6, two representative components of the exocyst complex, in OLs and myelin. By immunoblot analysis, Sec8 and Sec6 were detectable as unique bands at 110 kDa and 86 kDa, respectively, in OLs and astrocytes in culture, myelin and brain (Fig. 1A). Sec8 and Sec6 expression levels were not developmentally regulated during the OL lineage. Interestingly, Sec8 and Sec6 were present in purified myelin membranes, paralleling earlier observations of SNARE and Rab proteins in myelin (Madison et al., 1996; Madison et al., 1999).

We next analyzed the subcellular distribution of Sec8 in OLs in enriched cultures. Using double-label confocal immunofluorescence microscopy, we found that Sec8 was present in the soma, where it overlapped partially with the

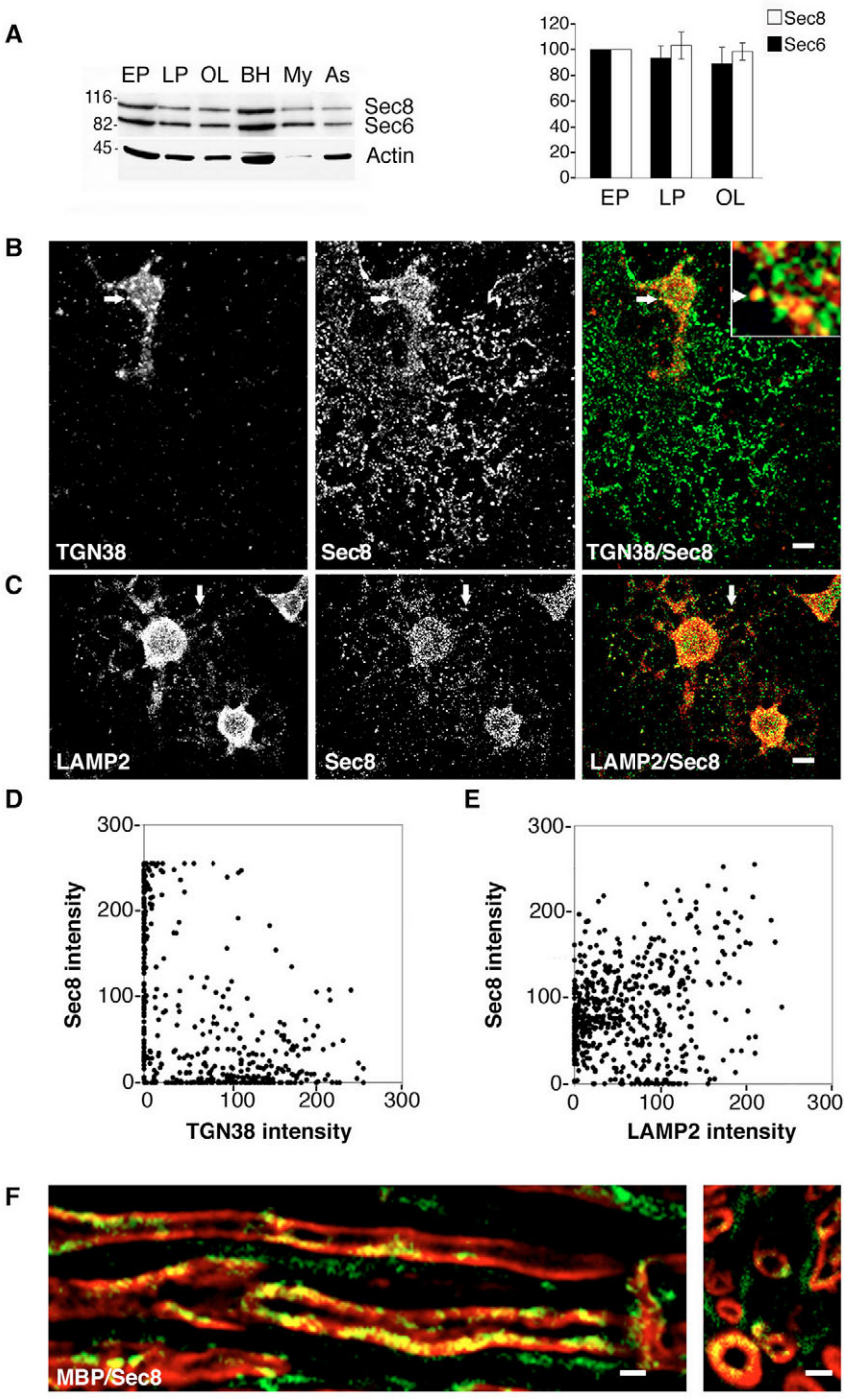

Fig. 1. The exocyst components Sec8 and Sec6 are present in OLs and myelin. (A) OL lineage cells, myelin, brain homogenate and astrocytes were analyzed by immunoblot (10 μg protein/lane). The levels of Sec8 and Sec6 were normalized to actin and quantified for n=3 independent OL cultures. (B-E) Sec8 (green) colocalization with (B) TGN38 (red) and (C) LAMP2 (red) in OL processes was assessed by confocal microscopy and ratiometric particle analysis for (D) 434 and (E) 560 randomly chosen puncta in eight representative OLs, two independent cultures per condition. (B) The arrows indicate colocalization between TGN38 and Sec8, shown at a higher magnification in the inset. (F) Sec8 (green) is detectable along MBP-positive (red) myelinated axons in mouse spinal cords sectioned longitudinally (left panel) or transversally (right panel). No immunofluorescence was detected when cells and/or sections were incubated with pre-immune IgGs and secondary Abs (data not shown). EP, early OL progenitors; LP, late OL progenitors; OL, mature OLs; BH, whole-brain homogenate; My, myelin; As, astrocytes. Bars, 10 μm (B,C), 2 μm (F).

trans-Golgi network (TGN) marker TGN38 (Fig. 1B inset, arrows) and with the endosomal marker LAMP2 (Fig. 1C). Sec8 was also present in the myelin-like membranes in terminally differentiated OLs (Fig. 1B) and in the processes of less mature OLs lacking membrane sheets (Fig. 1C, arrows). Sec8 was present in punctate structures, consistent with its proposed association with the vesicular transport pathway. Colocalization of Sec8 puncta with TGN38 or LAMP2 along OL processes was quantified by single-particle ratiometric analysis (Barbarese et al., 1995). For this, uniform background staining was subtracted in both channels and the intensities in the red (R) and green (G) channels associated with each well-resolved punctum were measured. Particles labeled exclusively in red are situated on the *x*-axis, whereas those labeled exclusively in green are on the *y*-axis; particles equally labeled in red and green are on the bisector line. We found that clearly resolved Sec8 puncta overlapped with ~28% of the rare TGN38-positive puncta (Fig. 1D) and with ~50% of the LAMP2-positive puncta (Fig. 1E). To assess the distribution of Sec8 in vivo we analyzed spinal cord sections by confocal immunofluorescence microscopy. Sec8 was present in clusters along longitudinal sections of MBP-positive myelinated axons (Fig. 1F, left panel) and labeled a fraction of the myelinated axons in cross-section (Fig. 1F, right panel).

In conclusion, in OLs Sec8 is associated both with a cytoplasmic compartment and with the myelin membrane, suggesting that, following vesicle fusion, Sec8 remains partly associated with its target membranes and is partly recruited to the recycling endosomal pathway.

Sec8 plays a role in OL differentiation and membrane formation

To determine the potential contribution of Sec8 to OL membrane formation, we overexpressed the protein in primary cultures of OLs. Progenitor cells were infected overnight with adenoviruses (AV) expressing either enhanced green fluorescent protein (EGFP) (EGFP-AV) (Fig. 2A) or a FLAG-tagged form of Sec8 (Sec8-AV) (Fig. 2B-D), with a ~70% infection efficiency. Cells were allowed to differentiate for 72 hours in defined medium, then fixed and double-labeled with antibodies (Abs) against FLAG to identify infected cells, and myelin basic protein (MBP) to identify mature OLs. Exogenous Sec8 was localized in the OL soma (Fig. 2B, arrows; 2C,D), processes (Fig. 2B,C) and myelin-like membranes (Fig. 2D), similar to the endogenous protein (see Fig. 1, above). By contrast, control EGFP was detected mainly in the cytoplasm by using the same image acquisition parameters (Fig. 2A). The number of MBP-positive mature OLs was modestly increased to 29±3% by Sec8-AV expression, compared with 19±5% for control EGFP-AV ($P<0.04$) (Fig. 2E). We further analyzed OL differentiation by dividing infected OLs into three categories according to their morphological complexities (see Materials and Methods): low (Fig. 2B), medium (Fig. 2C) and high (Fig. 2D). Sec8-AV expression significantly increased the number of mature OLs with highly complex morphology when compared with control EGFP-AV ($P_{high}<0.001$; $P_{medium}<0.017$; $P_{low}<0.15$) (Fig. 2F). Accordingly, the areas of OLs expressing Sec8-AV were larger than those of OLs in the control group (Fig. 2G).

To evaluate the effect of Sec8 on OL protein expression, infected cells were harvested and the lysates analyzed by immunoblot (Fig. 3). Protein levels in Sec8-AV-infected cells were compared with both control EGFP-AV- (Fig. 3A) or null-control-AV- (Fig. 3C) infected cells. In both cases, increased Sec8 levels were correlated with higher levels of MBP (~1.5-fold; $P<0.002$). We further analyzed and quantified a larger set of myelin proteins and found that an approximately twofold Sec8 overexpression resulted in significant increases in the levels of membrane proteins such as OSP/Claudin11 and myelin associated glycoprotein (MAG) (~twofold; $P<0.002$). By contrast, no significant changes were observed for 2,3-cyclic nucleotide 3-phosphodiesterase (CNP), an OL protein associated with the cytoskeleton (Dyer and Benjamins, 1989; Bifulco et al., 2002; Lee et al., 2005), CASK ($P>0.05$) or actin ($P>0.7$) (Fig. 3A,B). Sec6 levels were increased in some cases, but without reaching statistical significance ($P>0.7$). In conclusion, Sec8 overexpression led to an increase in the levels of a subset of myelin proteins, consistent with a higher membrane complexity as observed by immunofluorescence microscopy, and suggesting a general increase in membrane addition.

To test whether Sec8 is necessary for OL differentiation we analyzed the effect of small interference RNA (siRNA)-mediated Sec8 gene silencing in these cells. Progenitor cells were transfected with 50 nM siRNAs, allowed to differentiate for 48 hours and were analyzed by immunocytochemistry and immunoblot (Fig. 4). Transfection efficiency was assessed by fluorescence microscopy analysis of fluorescently tagged siGloCyclophilinB siRNAs (siGloCipB) (Fig. 4A). Transfected siRNAs were detectable as perinuclear (arrows) and nuclear fluorescent puncta in 60–70% of cells, whereas non-transfected siRNAs were occasionally detectable on plasma membranes. By immunoblot analysis, we found that Sec8 siRNAs reduced Sec8 protein levels by 60–75% compared with control siRNAs (Fig. 4B). Interestingly, the amount of MBP, protein expressed by mature OLs, was also significantly reduced, whereas no changes were seen in the levels of Sec6. By fluorescence immunocytochemistry we found that Sec8 siRNAs reduced the number of mature OLs 2.5-fold to threefold ($P<0.00002$) (Fig. 4C,D) and decreased the number of OLs with medium ($P<0.03$) and high ($P<0.01$) morphological complexity, whereas reciprocally increasing the number of those with low complexity ($P<0.0005$) (Fig. 4E). This was consistent with an observed delay in process formation and reduction in cell survival in OLs transfected with Sec8 siRNAs. We evaluated cell death by propidium iodide incorporation and found that survival was 58±5% for the cells transfected with Sec8 siRNAs compared with 80±5% for cells transfected with control siRNAs or non-transfected cells ($n=2000$ cells/condition, $n=6$ from three independent cultures, $P<0.006$). This suggests that Sec8 is a central player in OL vesicular transport and thus important for OL survival and morphological differentiation.

To test the role of Sec8 block-of-function in mature OLs, we co-injected either anti-Sec8 Ab or control IgG and dextran (used to identify injected OLs) into cells. One hour after microinjection, OLs were fixed, labeled with anti-MBP Ab and analyzed. In both conditions we found similar backgrounds of cells with fragmented processes and/or membranes (shown as group1) suggesting that anti-Sec8 Ab is not toxic for OLs. Interestingly, OLs injected with anti-Sec8 Ab had significantly

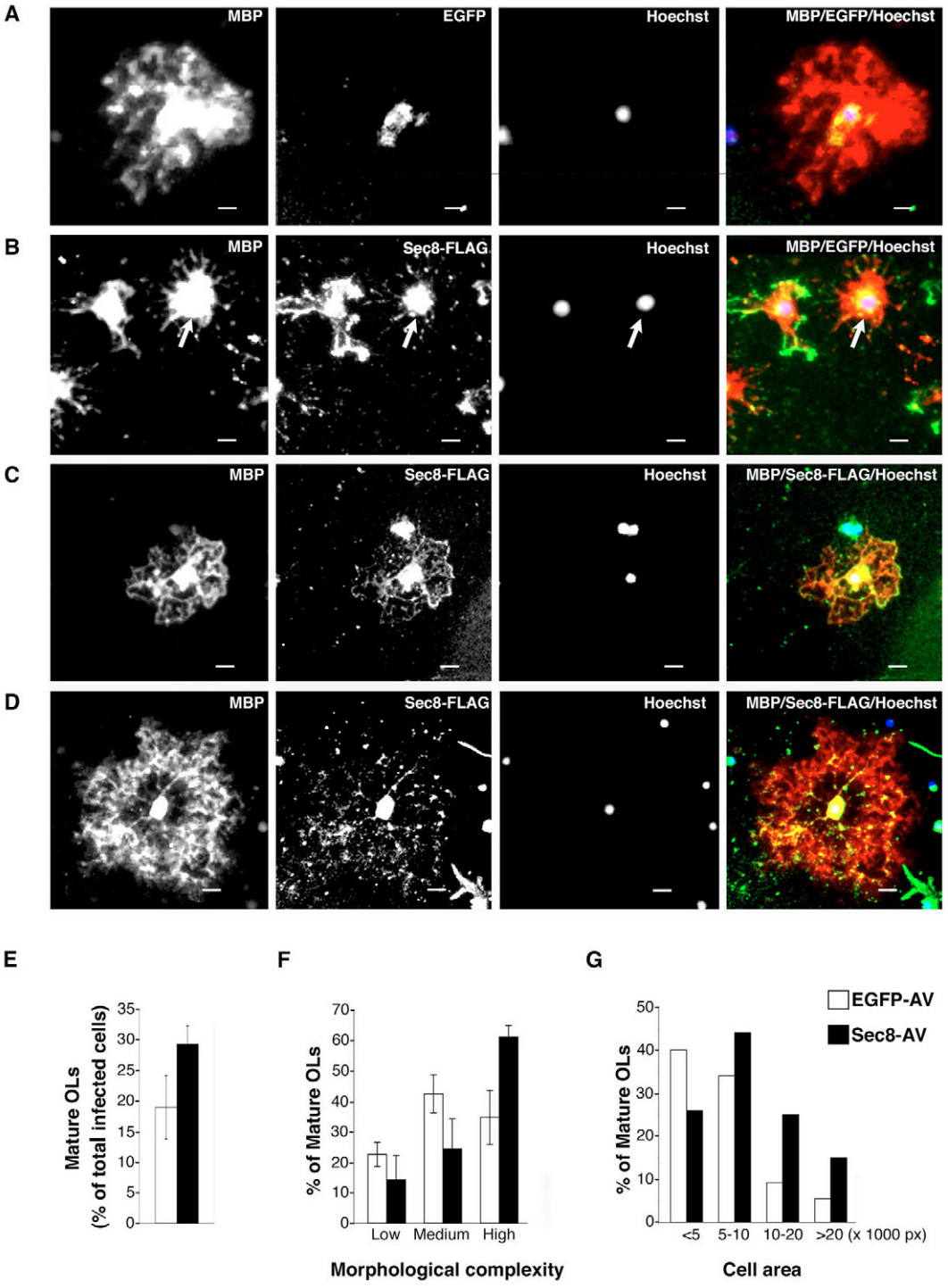

Fig. 2. Sec8 overexpression favors OL morphological differentiation. (A-D) OL progenitors were infected overnight with adenoviruses encoding (A) control EGFP (EGFP-AV) or (B-D) FLAG-tagged Sec8 (Sec8-AV), allowed to differentiate for 3 days, fixed, labeled with anti-MBP- (red) and anti-FLAG- (green) Abs and analyzed by epifluorescence microscopy. OLs were assigned to three morphological categories: (B) low, (C) medium or (D) high complexity. Cell bodies were overexposed in order to observe protein localization in the processes. Bars, 10 μm; px, pixels. (E) Mature MBP-positive cells were plotted as percentage of the total infected cells. (F) The number of OLs in each morphological category is shown as a percentage of the total MBP-positive OLs (500 cells/condition, n=6 from three independent cultures). (G) The areas of 100 randomly chosen MBP-positive cells per condition were measured and plotted. Only infected cells were analyzed. (E-G) White bars, control EGFP-AV; black bars, Sec8-AV infected.

smaller areas and shorter processes (shown as group 2 and corresponding to the low and medium morphological complexities described in Fig. 2) when compared with control mouse IgG-injected cells that had higher morphological complexities (shown as group 3). This suggests a role for Sec8 in cytoskeleton dynamics and membrane maintenance, in congruence with data suggesting a role for exocyst components in cytoskeleton modulation in other systems (Vega and Hsu, 2001; Sugihara et al., 2001; Wang et al., 2004; Aronov and Gerst, 2004). In summary, the three experimental approaches described above are consistent in indicating that Sec8 plays a central role in OL membrane formation and maintenance.

Sec8 associates with CASK and OSP/Claudin11

To identify some of the Sec8 binding partners in OLs, we used immunoprecipitation, subcellular fractionation and immunocytochemistry of enriched populations of OLs in culture (Fig. 6). We asked whether Sec8 associated in OLs with CASK, a multidomain scaffolding protein that is also involved in protein transport to the neuronal synapse and to the epithelial

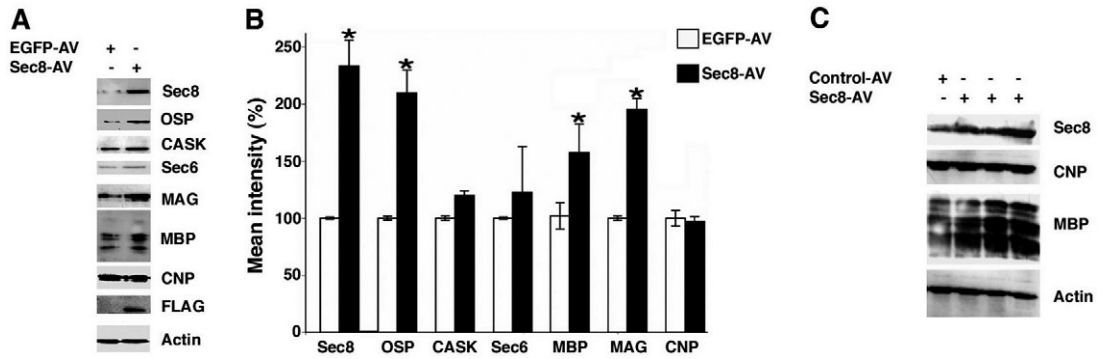

Fig. 3. Sec8 overexpression increases the levels of a subset of myelin proteins. OL progenitors were infected with Sec8-AV and either (A) control EGFP-AV or (C) control null-AV and allowed to differentiate for 3 days in defined medium. Cell lysates were analyzed by immunoblot for (A) Sec8, Sec6, OSP/Claudin11, CASK (10 μg protein/ lane); MAG, CNP, MBP, actin (2 μg protein/lane) and (B), and data are presented as percentage of control levels. (C) Cell lysates were analyzed for Sec8, MBP, CNP and actin (10 μg protein/lane). *n*=5 from three independent cultures, * indicates statistically significant differences.

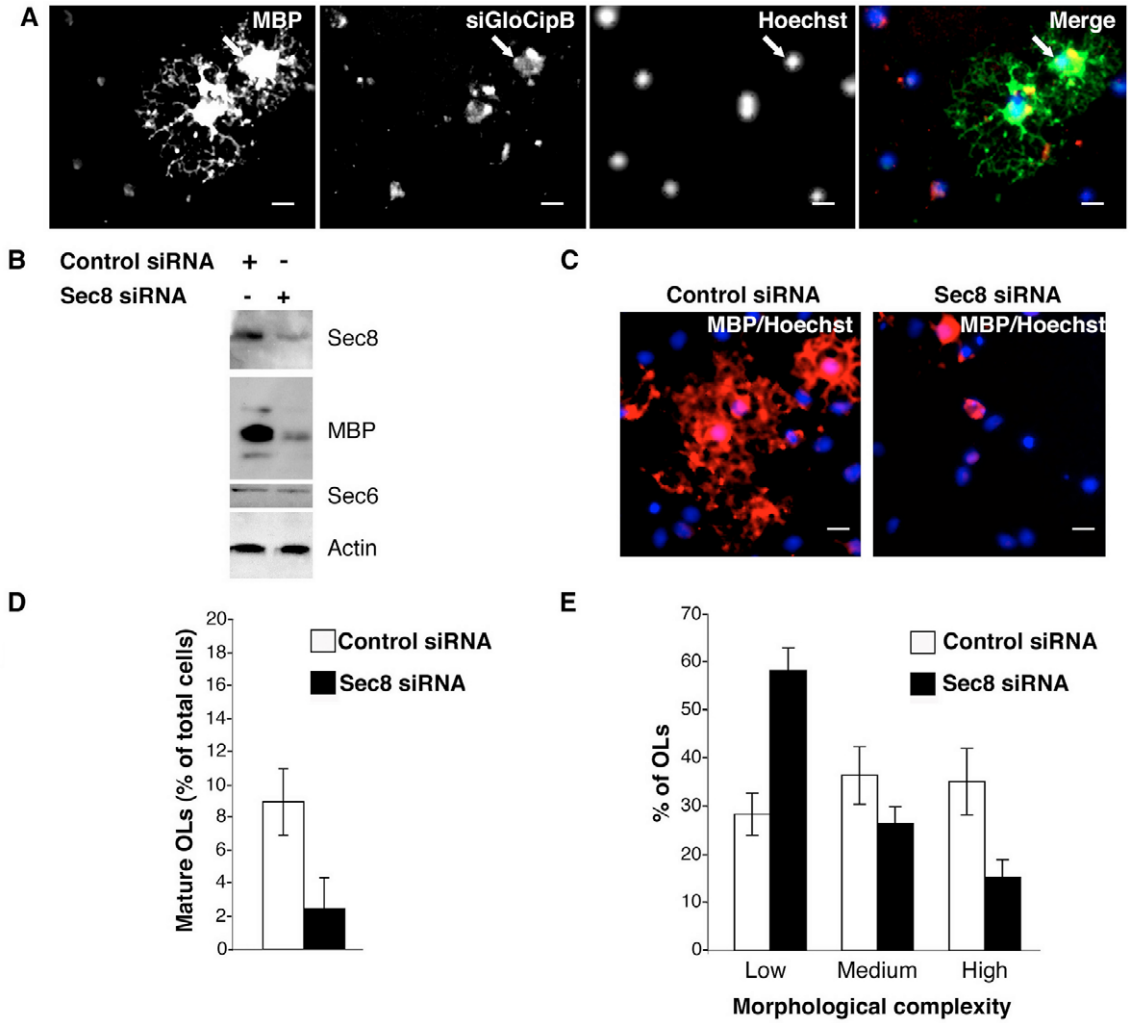

Fig. 4. siRNA-mediated interference with Sec8 expression decreases OL morphological differentiation. OL progenitors were transfected with (A) fluorescently tagged siRNAs (siGloCipB, red) and (B-E) Sec8 or control siRNAs. Arrows in A indicate a siGlo-positive perinuclear punctum. Cells were allowed to differentiate for 2 days and analyzed by (A,C) fluorescence microscopy for MBP and Hoechst or (B) immunoblot for Sec8, Sec6, MBP and actin (*n*=8 from four independent cultures). (D) The number of mature MBP-positive cells was plotted as a percentage of the total Hoechst-labeled nuclei (5000 cells/condition, *n*=11, four independent cultures). (E) Morphological complexity of CNP-positive OLs was analyzed for 900 cells/condition (*n*=6, two independent cultures). Bars, 10 μm.

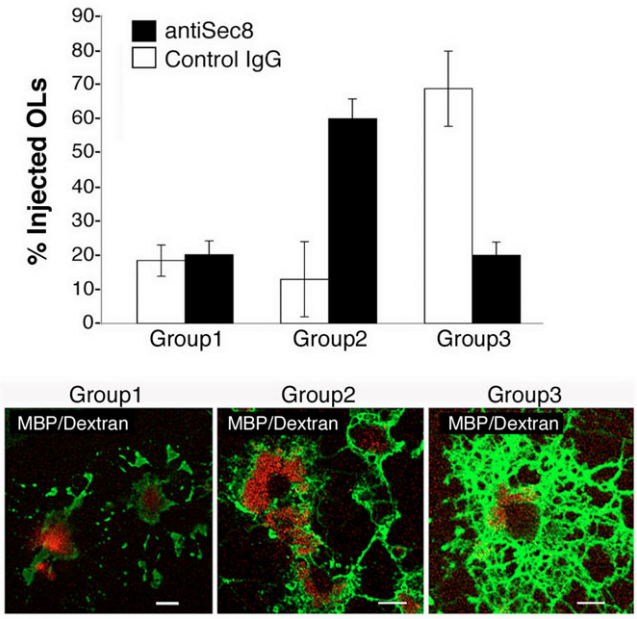

Fig. 5. Ab perturbation of Sec8 function significantly influences OL morphology. Mature OLs in culture were microinjected with dextran (red) and either anti-Sec8 Ab or IgG; 60 minutes after injection, cells were fixed and labeled with anti-MBP Ab (green). Cells were analyzed into three groups: (a) group 1: cells with fragmented processes and/or membranes; (b) group 2: cells with small areas and short processes (low and medium morphological complexity) and (c) group 3: cells with high complexity. Cells in each group are shown as percentage of the total dextran-positive injected cells (173 cells injected with anti-Sec8 Ab; 112 cells injected with IgG; n=3 independent experiments; P<0.003). Bars, 10 μm.

basolateral membrane (see Introduction). We found that, indeed, anti-Sec8 Ab coimmunoprecipitated CASK and Sec6 – a known Sec8 binding partner – at similar levels, and that anti-CASK Ab coimmunoprecipitated Sec8 (Fig. 6A).

Interestingly, Sec8 and, to a lower degree, CASK, were coimmunoprecipitated (both from OL and brain lysates) with a major myelin protein, OSP/Claudin11, but not with myelin OL protein (MOG) (Fig. 6A). Since a significant amount of OSP/Claudin11, but not MOG, associates in OLs with glycosphingolipid/cholesterol-enriched membrane domains (also known as lipid rafts) (Kim and Pfeiffer, 1999; Marta et al., 2003; Marta et al., 2005), we asked whether Sec8 and CASK are present in this membrane fraction (Fig. 6B). For this, OL post-nuclear supernatants were extracted with 1% Triton X-100 (30 minutes, 4°C) and the insoluble fraction was floated on a 5-35% sucrose gradient prior to immunoblot analysis. We found that a significant proportion of total Sec8 and CASK were insoluble in these conditions and that they cofractionated with OSP/Claudin11 at low density on sucrose gradients. These proteins were also detectable in the heavier fractions, probably the reflection of interactions with cytoskeletal elements (Kim and Pfeiffer, 1999).

To analyze the relative localizations of OSP/Claudin11, CASK and Sec8, we used confocal double-imunofluorescence microscopy and ratiometric analysis of fluorescently labeled puncta (Fig. 6C-E, arrows). In OL processes and myelin-like membranes, OSP/Claudin11 colocalized with CASK in ~65% of the analyzed puncta (Fig. 6C) and with Sec8 in ~60% of the puncta (Fig. 6D). By contrast, Sec8 colocalized with MOG in only ~30% of the puncta (Fig. 6E). Owing to technical difficulties (both Abs were produced in mouse), we did not directly study the relative localization of Sec8 and CASK. In conclusion, Sec8 and CASK might specifically regulate OSP/Claudin11 recruitment to the myelin membrane.

CASK is associated with motile vesicle-like structures in OLs

To determine whether CASK, identified as a Sec8 binding partner, was indeed associated with transport vesicles, we transfected OL progenitors with CASK-EGFP and analyzed live OLs by time-lapse microscopy (Fig. 7A,B). We chose to analyze CASK-EGFP because its intracellular localization is similar to that of the endogenous protein, whereas Sec8-EGFP fails to associate with its target membranes (Matern et al., 2001; Maximov and Bezprozvanny, 2002). CASK-EGFP adopted a punctate pattern in OL processes, similar to the endogenous protein, even though it was overexpressed (Fig. 7A). One representative time-lapse series for CASK-EGFP dynamics is shown in Fig. 7B and in supplementary material Movie 1. We observed two classes of puncta: (1) large (0.8-2 μm), relatively stationary (velocities <0.05 μm/second), associated mainly with branching points, and (2) small (0.2-0.4 μm) mobile structures (arrows) that moved with velocities of 0.2-0.25 μm/second. Similar dynamics have been previously described for motor-driven transport vesicles and RNA granules in various cell types, including OLs (Ainger et al., 1993; Hirokawa and Takemura, 2005; Zaliapin et al., 2005). It remains to be investigated how many of these puncta colocalize with Sec8 and OSP/Claudin11. To further understand the nature of CASK puncta in OL processes, we used fluorescence confocal microscopy and particle ratiometric analysis to study its localization relative to dynein, a molecular motor associated with transport vesicles. CASK was detectable in approximately 80% of the dynein-positive puncta (Fig. 7C, arrows). In neurons, CASK associates with transport vesicles as part of a larger protein complex that includes Mint1 (Setou et al., 2000). The interaction between Mint1 and CASK appears to be conserved in OLs, because Mint1 was coimmunoprecipitated by anti-CASK Ab in these cells (supplementary material Fig. S1). Together, these data suggest that CASK is associated with motor-driven transport vesicles in OL processes.

CASK expression and localization were also analyzed by confocal immunofluorescence microscopy in myelinating OL-neuronal cocultures, brain and spinal cord (Fig. 8). In cocultures, CASK was present in MBP-positive OLs (Fig. 8A, arrows) and also along the MBP-positive internodes of myelinated fibers (Fig. 8A, arrowheads). In vivo, anti-CASK Ab also labeled MBP-positive cells (Fig. 8B) and MBP-positive myelinated axons (Fig. 8C). The arrows indicate CASK localization near the plasma membranes that separate two adjacent cells (Fig. 8) and CASK presence in a myelinated fiber in cross-section (Fig. 8C). In conclusion, we show that key multidomain-proteins (e.g. CASK, Mint1) are present in OLs where they might play a role in protein trafficking to the myelin membranes.

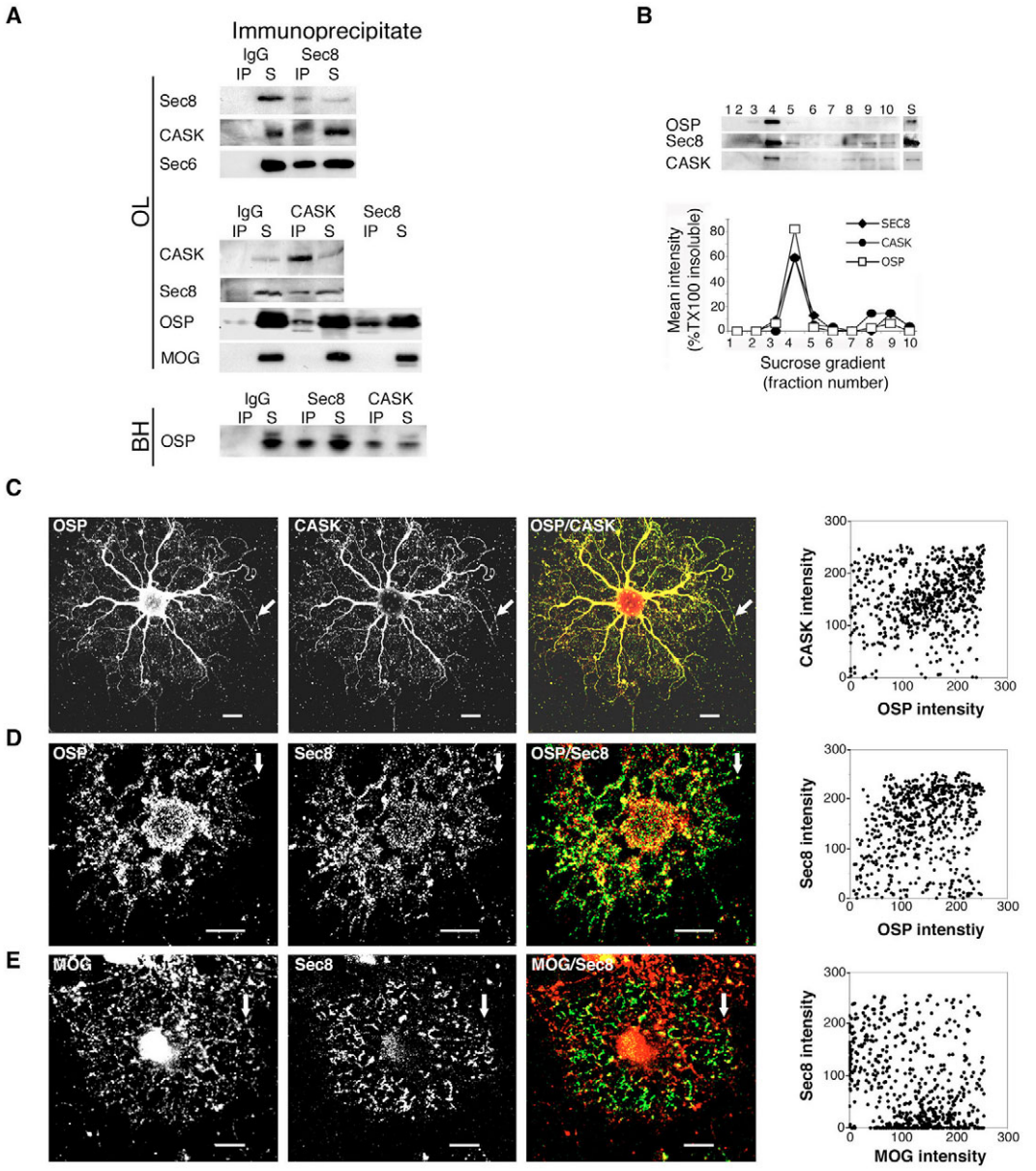

Fig. 6. Sec8 associates with CASK and OSP/Claudin11 in OLs. (A) Sec8, CASK and OSP/Claudin11 coimmunoprecipitate. Mature OL lysates were incubated with anti-Sec8 Ab (2E12), anti-CASK Ab or pre-immune IgG and immunoprecipitates were analyzed by immunoblot. IP, immunoprecipitate; S, 10% of the supernatant fraction. (B) OSP/Claudin11, Sec8 and CASK cofractionate and float to low densities on sucrose gradients. The 1% Triton X-100 insoluble pellet fractions were isolated from mature OL lysates at 4°C, floated on sucrose-step gradients and analyzed by immunoblot; S, 15% of the soluble fraction. (C-E) Colocalization of (C) CASK (green) and OSP/Claudin11 (red), (D) Sec8 (green) and OSP/Claudin11 (red) or (E) Sec8 (green) and MOG (red) in OL processes was analyzed by confocal microscopy and ratiometric particle analysis for ~680 randomly chosen puncta in 5-7 representative cells, three independent cultures. The Abs used for colocalization analysis were against the intracellular C-terminal regions of OSP/Claudin11 and MOG. Bars, 10 μm. Arrows indicate fluorescent puncta positive for OSP and CASK (C), positive for OSP and Sec8 (D), or positive for MOG, but not for Sec8 (E).

Discussion

Eukaryotic cell growth and differentiation depend on the precise regulation of vesicle transport, a highly conserved mechanism for inserting additional membrane to the plasmalemma (Nelson, 2003; Jahn, 2004). Thus, protein complexes that control vectorial trafficking and capture of transport vesicles to their target membranes are expected to be essential for OL differentiation, a process characterized by rapid process extension, membrane expansion and polarization (Colman et al., 1982; Pfeiffer et al., 1993; Madison et al., 1999). It has been suggested that myelin proteins and lipids are added at the 'trailing edge' of the myelin spiral (Gould et al., 1977), but the molecular players and the interactions involved in these processes are not well understood.

In the present study, we provide evidence that Sec8 plays a central role in OL membrane formation. Thus, Sec8 overexpression favored the formation of mature OLs with high morphological complexity and large myelin-like membranes, concomitantly increasing the expression of a subset of myelin membrane proteins. Reciprocally, siRNA-mediated interference with Sec8 expression resulted in delayed OL differentiation and decreased OL morphological complexity, and intracellular Ab-mediated blocking of Sec8 induced a reduction in OL cell area. It is likely that interference with Sec8 function perturbs vesicle capture to the membrane and influences cytoskeletal dynamics (Grindstaff et al., 1998; Vega and Hsu, 2001; Sugihara et al., 2002; Wang et al., 2004; Tsuboi et al., 2005), leading to a delay in membrane addition and consequently in OL differentiation. Together these data provide a pattern consistent with the hypothesis that in OLs Sec8 is instrumental for exocytosis and thus for process growth and myelin-membrane-formation and -maintenance.

Components of the exocyst, including Sec8, play a role not only in the recruitment of transport vesicles to the plasma membranes, but also in early secretory events (e.g. protein translation, translocation) in yeast, epithelial cells and neurons

(Toikkanen et al., 2003; Lipschutz et al., 2003; Sans et al., 2003). In epithelial cells, for example, exocyst components stimulate the synthesis of basolateral but not apical proteins, and subsequently favor membrane addition to the epithelial basolateral compartment during tubulogenesis (Lipschutz et al., 2000; Lipschutz et al., 2003). In OLs, Sec8 might be involved in a similar pathway, that coordinates early events in protein synthesis with the exocytic machinery for a specific subset of proteins, including MBP and MAG, but not CNP; differential regulation of these proteins has been suggested to play a role in myelin membrane formation and maturation (Pfeiffer et al., 1981; Campagnoni et al., 1991). Sec8 localization to the endoplasmic reticulum in OLs (data not shown) suggests a role for this protein during early steps in the secretory pathway, but further experiments are required to understand its potential function (e.g. pre- versus post-translational events).

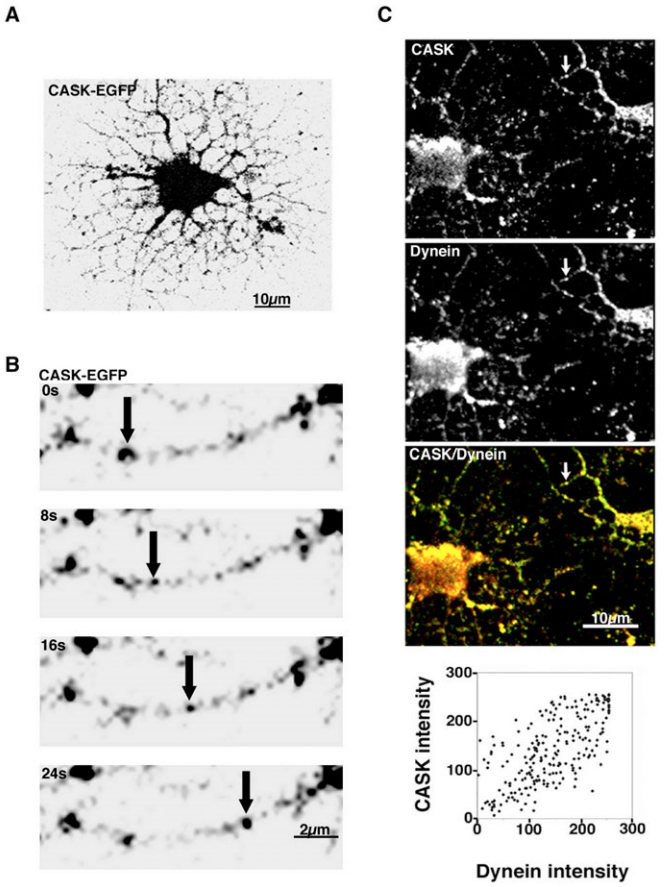

Fig. 7. CASK is present in mobile vesicle-like structures in OLs. (A,B) OL progenitors in culture were transfected with a CASK-EGFP fusion protein and OLs were analyzed by confocal microscopy after 3 days. Images were converted to grayscale mode and colors inverted; CASK-EGFP is shown in black. (B) Time-lapse analysis of CASK-EGFP in a representative OL process segment shows that the protein is present in mobile puncta (arrows). Images were collected every 8 seconds. (C) OLs in culture labeled with anti-CASK Ab (green) and anti-dynein Ab (red) were analyzed by confocal microscopy and colocalization in OL processes was quantified for 280 puncta in eight representative cells from two independent cultures. Arrows indicate a fluorescent punctum positive for CASK and dynein. Bars, 10 μm (A,C), 2 μm (B).

Sec6, another component of the exocyst complex, is also present in OLs and myelin. Consistent with previous studies in other systems (Terbush et al., 1996; Vega and Hsu, 2001; Moskalenko et al., 2003), Sec6 coimmunoprecipitates with Sec8 in OLs, suggesting they associate during transport. However, compared with Sec8, Sec6 displays a stronger association with a higher density, tubulin-enriched subcellular fraction (data not shown). In addition, anti-CASK Ab coimmunoprecipitates Sec8 well, but Sec6 only weakly (data not shown), consistent with distinct Sec8 and Sec6 profiles identified in various systems by coimmunoprecipitation (Sans et al., 2003; Beronja et al., 2005), co-sedimentation (Beronja et al., 2005) and sub-cellular distribution (Vik-Mo et al., 2003; Beronja et al., 2005). Together, these data suggest that subpopulations of Sec8 and Sec6 become part of the same complex during transport, although they might also have distinct functions. It should prove interesting for future studies to characterize Sec6 function in OLs.

We have also identified additional components of the OL transport machinery, including the multidomain scaffolding proteins CASK and Mint1. Sec8 and the CASK/Mint1 complex are involved in NMDA receptor transport to the neuronal synapse (Setou et al., 2000; Fallon et al., 2002; Sans et al., 2003). We describe for the first time an association between CASK and Sec8 in OLs. This association is likely to occur as part of a larger complex, which might link the vesicle-associated complex with either molecular motors that drive vesicle transport along microtubules or with the vesicle-docking and fusion-machinery, as previously shown in neurons (Setou et al., 2000; Biederer and Sudhof, 2000).

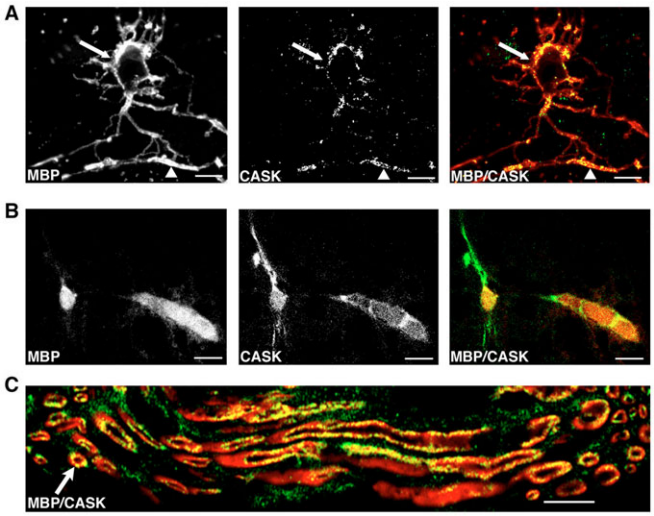

Fig. 8. CASK is detectable in MBP-positive OLs and myelinated axons in cocultures and in vivo. (A) OL-neuronal cocultures were immunolabeled with anti-MBP Ab (red) and anti-CASK Ab (green), and analyzed by confocal microscopy followed by the 3D reconstruction of *Z*-stacks of images. Arrows indicate MBP-positive OLs and arrowheads indicate myelinated fibers. (B,C) CASK (green) is detectable in (B) cells with a chain-like pattern, suggestive of intrafascicular OLs (rat brain) and (C) MBP-positive myelinated fibers in longitudinal or cross-sections (mouse spinal cord). The arrow in (C) indicates a CASK-positive cluster localized in an MBP-positive myelinated fiber in cross-section. Bars, 10 μm (A-C), 5 μm (C).

Sec8 and CASK specifically associate with the major myelin protein OSP/Claudin11 and may play a role in the recruitment of OSP/Claudin11 to the myelin membrane. OSP/Claudin11 is currently the only identified component of the tight junctions that are present along myelin internodes (Dermietzel, 1980; Mugnaini and Schnapp, 1974; Bronstein et al., 1996; Bronstein et al., 1997; Morita et al., 1999; Gow et al., 1999; Tiwari-Woodruff et al., 2001; Chow et al., 2005). The function of these junctions in myelin is not entirely clear, but it can be hypothesized, by analogy with other cell types (Grindstaff et al., 1998; Fanning et al., 1998; Zahraoui et al., 2000; Poliak et al., 2002), that they serve as active organizing centers for vesicle transport and membrane growth during myelin biogenesis. Finally, Sec8 and CASK associate with OSP/Claudin11 in detergent-insoluble glycosphingolipid-cholesterol-enriched microdomains (lipid rafts), structures involved in protein trafficking and membrane polarization (Simons and Ikonen, 1997; Nusrat et al., 2000) that may be of particular importance for myelin membrane formation (Kim et al., 1995; Simons et al., 2000; Taylor et al., 2002; Schneider et al., 2005). Similar to other cell types (Inoue et al., 2003; Salaun et al., 2004), lipid rafts might play a role in exocyst-dependent vesicle recruitment to the myelin membrane.

Little is known about the localization and function of exocyst components in peripheral myelin. This will constitute an interesting avenue for future studies, especially in view of the potential association of Sec8 with multidomain proteins and/or claudins (Itoh et al., 1999; Riefler et al., 2003; Sans et al., 2003; Yeaman et al., 2004), molecules localized at junctional specializations present at the Schmidt-Lanterman incisures, Schwann cell microvilli and paranodal loops, and suggested to play a role in peripheral myelin addition and remodeling (Mugnaini and Schnapp, 1974; Tezlaff et al., 1978; Poliak et al., 2002; Salzer, 2003; Bolino et al., 2004). Further, an in vivo functional analysis of the potential role of Sec8 in myelination would be informative but is technically difficult at this point. A Sec8 functional deletion is embryonic lethal (Friedrich et al., 1997), and the development of animal models with conditional Sec8 deletions in specific cell types (e.g. OLs) may also be problematic because recent studies in *Drosophila* suggest that multiple exocyst components are essential for cell viability (Andrews et al., 2002; Murthy et al., 2003; Murthy et al., 2005).

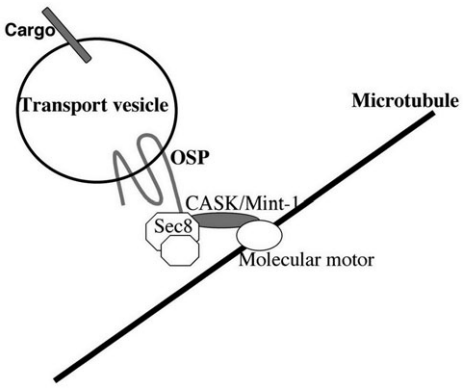

Fig. 9. Model. Sec8 associates with the CASK/Mint1 complex and mediates the transport of motor-driven vesicles carrying lipid- and protein-cargoes (e.g. OSP/Claudin11) along microtubules to the OL and myelin membranes.

In summary, we propose a model (Fig. 9), in which key trafficking molecules, including Sec8, CASK and Mint1, are part of a complex that regulates the transport of vesicles carrying lipid and protein cargoes (e.g. OSP/Claudin11) along cytoskeletal elements to the myelin membranes. In this way, these targeting proteins might play important roles in OL maturation, in the progression from initial OL-neuronal adhesion to myelin formation and in myelin membrane maintenance and repair.

Materials and Methods

OL cultures

Enriched OL cultures were isolated from neonatal rat telencephalon postnatal day (P) 0-2. Stage-specific enriched OL populations were grown first (a) in mN2 medium supplemented with 10 ng/ml platelet-derived growth factor (PDGF) and fibroblast growth factor 2 (FGF2) (both Amgen) for 72 hours, to obtain early progenitors, followed by culturing in (b) mN2 medium supplemented with 10 ng/ml FGF2 for 48 hours, to obtain late progenitors and were finally (c) further allowed to differentiate without growth factors to obtain mature OLs (Gard and Pfeiffer, 1993; Bottenstein and Sato, 1979; Bansal et al., 1996). Myelinating OL-neuronal cocultures were isolated from mouse brains (embryonic day 15), grown in defined medium and analyzed after 3 weeks (Lubetzki et al., 1993).

Tissue sections

Animals were perfused with 4% paraformaldehyde (PFA) (10 ml/animal) followed by PBS (10 ml/animal). Isolated rat brains (P 10-12) or mouse spinal cords (P 24) were fixed in 4% PFA in PBS (30 minutes, 4°C), cryo-protected in 20% sucrose overnight at 4°C, frozen in Tissue-Tek (Sakura FineTek), cryosectioned (10-15 μm) and post-fixed with 4% PFA (15 minutes, room temperature). Myelin purification was performed as described in Menon et al. (Menon et al., 2003).

Antibodies

Antibodies (Abs) used were (a) mouse monoclonal Abs against CASK, rSec8 (BD Biosciences), 2E12 (S. C. Hsu, NJ), Sec6 (Stressgen), MOG (C. Linington, Scotland), FLAG and actin (Sigma); (b) rat monoclonal Abs against LAMP2 (QED Biosciences); (c) rabbit polyclonal Abs against dynein (E. Barbarese, Farmington), OSP/Claudin11 (J. M. Bronstein, CA; S. Tsukita, Japan), Mint1 (Synaptic Systems), TGN38 (B. Eipper, CT), MOG (C. Linington), CNP (in house), MBP (Sternberger Monoclonals); (d) secondary Abs conjugated with Alexa-Fluor-488™ or -596 (Molecular Probes) and (e) nuclear dye Hoechst 33342.

Immunocytochemistry

Cells were fixed (4% PFA, 15 minutes, room temperature), permeabilized (0.1% Triton X-100, 5 minutes, 4°C), blocked (10% BSA, 1 hour, 4°C), incubated with primary (1 hour, 4°C) and secondary Abs (20 minutes, 4°C). Tissue was permeabilized (70% ethanol, 15 minutes, room temperature), blocked (10% BSA, 2 hours, room temperature), incubated with primary (overnight, 4°C) and secondary Abs (2 hours, room temperature). All samples were imaged in DABCO medium (250 mg of 1,4-diazobicyclo-(2,2,2)- octane, 9 ml glycerol, 1 ml PBS pH 8.6).

Immunoblot analysis

OLs were harvested in 1×RIPA buffer (10 mM Tris pH 7.6, 0.01% SDS, 1% Na deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM EGTA) with protease inhibitors [1 mM phenylmethylsulphonylfluoride (PMSF), 10 μg/ml leupeptin/aprotenin; Sigma], homogenized (27G needle), centrifuged (5 minutes, 5000 ***g***), and post-nuclear supernatants or cell lysates run on SDS-PAGE, transferred to a nitrocellulose membrane which was blocked in 5% non-fat milk in 0.5% Tween 20 (Fisher Scientific) (1 hour, room temperature), incubated with the primary (overnight, 4°C) and the secondary (30 minutes, room temperature) Abs. The signal was detected using enhanced chemo-luminescence (ECL, Amersham Biosciences). The mean intensities of the bands were measured (NIH Image1.61), analyzed and shown as mean value ± s.d. (ANOVA single factor).

Immunoprecipitation

Mature OLs were harvested in 0.5×RIPA buffer with 0.5% Triton X-100, homogenized, pre-cleared by centrifugation (30 minutes; 13,000 ***g***), pre-cleared with Protein G beads (Amersham Biosciences) (overnight, 4°C). Lysates (1 μg/ μl) were incubated with Abs (4-8 μg/ml, overnight, 4°C) and then with Protein G beads (2 hours, 4°C). Beads were washed in lysis buffer and protein samples solubilized with reducing or non-reducing Laemmli buffer and run on SDS-PAGE. Each experiment was repeated at least three times.

Sucrose-gradient ultracentrifugation

OL post-nuclear supernatants were extracted with 1% Triton X-100 in lysis buffer (15 mM NaCl, 50 mM EDTA, 250 mM Tris, protease inhibitors) for 30 minutes at

4°C, centrifuged, and pellets resuspended in 500 μl extraction buffer. This was mixed with 1 ml of 2 M sucrose and overlaid with 2 ml of 1 M and 1.5 ml of 0.2 M sucrose. Samples were centrifuged (16-18 hours; 200,000 ***g***; 4°C) and 0.5 ml fractions removed from the top of the tube. Fraction density was measured by refractometry. Protein was concentrated by ethanol precipitation (overnight, –20°C) and pellets solubilized and run on SDS-PAGE (Kim and Pfeiffer, 1999; Taylor et al., 2002).

Confocal microscopy and time-lapse analysis

Confocal microscopy analysis was done with a Zeiss 410 microscope with a 63×, 1.4 or 1.25 NA, plan-apochromatic objective (Carl Zeiss). Dual-channel images were simultaneously collected using dual excitation at 488 nm and 568 nm with appropriate emission filters, merged and analyzed with Adobe Photoshop 7 and 3D Imager. Colocalization was measured by ratiometric particle analysis using NIH Image 1.61 (Barbarese et al., 1995). Time-lapse analysis was performed at room temperature and individual particle movement analyzed with Metamorph 6.1 (Universal Imaging Co.).

Transfection

Transfection of CASK-EGFP (Maximov et al., 2002) and EGFP (R. Mains, CT) constructs was done using Gene Porter2 (Gene Therapy Systems) with 2-5% efficiency. Sec8-FLAG, control null-pShuttle (Ewart et al., 2005) or EGFP (Thiagarajan et al., 2004) adenoviruses were diluted in mN2 (10^6-10^7 plaque forming units/ml) then added to OLs for an overnight incubation.

Microinjection

Microinjection of OLs was as described in Shan et al. (Shan et al., 2003). We injected 10^{-13}-10^{-15} l of solution/cell, with concentrations of 0.125 μg/μl for Abs and 5 μg/μl for dextran Alexa Fluor 594 (10,000 MW, Molecular Probes).

siRNA experiments

siRNAs (Dharmacon): four pooled SMART-selected siRNAs that target rat Sec8; four pooled control non-targeting siRNA (two of which target firefly luciferase, all have ⩾four mismatches with all known rat genes); fluorescently tagged siGloCyclophilinB siRNAs. JetSI reagent (Qbiogene) was used for siRNA transfection at 50 nM final concentration.

Morphological complexity

Morphological complexity was assessed as described (Sperber and McMorris, 2001). OLs were assigned to three categories. (1) low: small areas, minimal development of secondary and tertiary processes; (2) medium: medium areas, moderate secondary and tertiary processes, radial symmetry; (3) high: large areas, extensive of secondary and tertiary processes, radial symmetry. We counted cells from six to eight fields/well; *n* indicates the number of wells unless otherwise stated. Data are shown as mean value ± s.d. (Anova single factor).

Cell death

Cell death was assessed by staining with 10 μg/ml propidium iodide (Molecular Probes; 10 minutes, 37°C) (Hewett et al., 2000).

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