Expression of Distinct Splice Variants of the Stem Cell Marker Prominin-1 (CD133) in Glial Cells

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KEY WORDS

prominin; oligodendrocyte; astrocyte; myelin sheath; membrane protrusion

ABSTRACT

Prominin-1 (CD133) is a cholesterol-interacting pentaspan membrane glycoprotein specifically associated with plasma membrane protrusions. Prominin-1 is expressed by various stem and progenitor cells, notably neuroepithelial progenitors found in the developing embryonic brain. Here, we further investigated its expression in the murine brain. Biochemical analyses of brain membranes at early stages of development revealed the expression of two distinct splice variants of prominin-1, s1 and s3, which have different cytoplasmic C-terminal domains. The relative abundance of the s3 variant increased toward adulthood, whereas the opposite was observed for the s1 variant. Our combined in situ hybridization and immunohistochemistry revealed the expression of prominin-1 in a subpopulation of Olig-2positive oligodendroglial cells present within white matter tracts of postnatal and adult brain. Furthermore, immunohistological and biochemical characterization suggested strongly that the s3 variant is a novel component of myelin. Consistent with this, the expression of prominin-1.s3 was significantly reduced in the brain of myelin-deficient mice. Finally, oligodendrocytes expressed selectively the s3 variant whereas GFAP-positive astrocytes expressed the s1 variant in primary glial cell cultures derived from embryonic brains. Collectively, our data demonstrate a complex expression pattern of prominin-1 molecules in developing adult brain. Given that prominin-1 is thought to act as an organizer of plasma membrane protrusions, they further suggest that a specific prominin-1 splice variant might play a role in morphogenesis and/or maintenance of the myelin sheath. © 2008 Wiley-Liss, Inc.

INTRODUCTION

Prominins are an emerging family of glycoproteins that among the multispan membrane proteins display a characteristic membrane topology (Fargeas et al., 2003a). Prominin-1 (CD133) contains five membranespanning domains, with an N-terminal domain exposed to the extracellular space followed by four alternating, small cytoplasmic and large glycosylated extracellular loops, and a cytoplasmic C-terminal domain (Corbeil et al., 1998; Miraglia et al., 1997; Weigmann et al., 1997) (for review see Fargeas et al., 2006). Prominin-1 transcripts show a great propensity to be alternatively spliced, thus giving rise to a large collection of prominin-1 proteins. Up to now, 12 alternative splicing affecting the open reading frame of mammalian prominin-1 were found in the three extracellular domains or mostly within the cytoplasmic C-terminal domain (Fargeas et al., 2003b, 2007). Indeed, either intron retention or exon skipping or the usage of a cryptic acceptor site might generate six different C-terminus tails (Fargeas et al., 2004).

The general interest in prominin-1 has grown rapidly, since it appears to be an important cell surface marker widely used to identify and isolate stem cells from various sources including the hematopoietic system (Corbeil et al., 2000; Richardson et al., 2004; Yin et al., 1997; Yu et al., 2002) (for review see Bauer et al., 2008). Prominin-1 is also expressed in embryonic stem cell-derived progenitors (Kania et al., 2005), and in several embryonic and adult epithelial cells (Corbeil et al., 2000, 2001a; Florek et al., 2005; Immervoll et al., 2008; Jászai et al., 2007a; Karbanová et al., 2008; Lardom et al., 2008; Weigmann et al., 1997) as well as in epithelialderived cells such as photoreceptor cells (Jászai et al., 2007b; Maw et al., 2000). In the central nervous system, prominin-1 was initially detected in neuroepithelial progenitor cells and the ependymal cells (Weigmann et al., 1997). The latter cells might represent one source of adult neural stem cells (Coskun et al., 2008). Prominin-

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1-positive progenitor cells have been successfully isolated from human fetal and *postmortem* brain (Schwartz et al., 2003; Tamaki et al., 2002; Uchida et al., 2000). Similarly, prominin-1-positive stem cells, which are lacking neuronal and glial lineage markers, could be purified from mouse postnatal cerebellum, and were shown to form self-renewing neurospheres, differentiate into astrocytes, oligodendrocytes, and neurons *in vitro* and, remarkably, generate each of these lineages after transplantation into the cerebellum (Lee et al., 2005). Thus prominin-1-positive stem cells may be important clinically, particularly with regard to bone marrow transplantation and brain injury/disease (Bornhäuser et al., 2005; Freund et al., 2006a; Kobari et al., 2001; Lee et al., 2005).

Prominin-1 has been detected in several malignant hematopoietic diseases (Baersch et al., 1999; Buhring et al., 1999; Green et al., 2000; Waller et al., 1999; Wuchter et al., 2001) as well as in solid tumors such as those derived from the brain, kidney, and colon (Bao et al., 2006; Florek et al., 2005; Hemmati et al., 2003; O'brien et al., 2007; Pfenninger et al., 2007; Ricci-Vitiani et al., 2007; Singh et al., 2003). Interestingly, it was recently demonstrated that prominin-1-positive human brain tumor cell fraction (as opposed to prominin-1-negative) contains cells that initiate tumor formation in immunodeficient mice (Singh et al., 2004), raising the exciting possibility that prominin-1 becomes a molecular target for effective cancer therapies.

The cell biological characterization of prominin-1 has revealed that this pentaspan membrane protein is selectively associated with the microvillus (Corbeil et al., 1999; Weigmann et al., 1997), primary cilium (Dubreuil et al., 2007; Florek et al., 2007), and other plasma membrane protrusions (Freund et al., 2006b; Giebel et al., 2004; Maw et al., 2000) irrespective of the cell type (for review see Corbeil et al., 2001b). The molecular mechanism underlying its microvillar retention involves a cholesterol-based membrane microdomain in which prominin-1 binds directly to plasma membrane cholesterol (Röper et al., 2000). Although no precise physiological function has yet been ascribed to prominin-1, the following observations, that is, (i) its general preference for plasma membrane protrusions (Weigmann et al., 1997), (ii) its association with a cholesterol-based membrane microdomain (Röper et al., 2000), and (iii) the retinal degeneration associated with a mutation in the human PROMININ-1 gene (Maw et al., 2000), have led to the hypothesis that prominin-1 may play a role in the biogenesis and/or functional maintenance of plasma membrane protrusions (Corbeil et al., 2001b). Specifically, prominin-1 may endow plasma membrane protrusions with an appropriate lipid composition and/or organization (Janich and Corbeil, 2007). Understanding the role of prominin-1 thus appears to be important since any alteration in its structure may be a cause of, as yet unexplained, human pathology in which plasma membrane protrusions containing prominin-1 are involved.

It has been reported that myelin, which is a highly specialized multilamellar plasma membrane protrusion of oligodendrocytes in the central (CNS) and Schwann cells in the peripheral (PNS) nervous system, also contains cholesterol-based membrane microdomains (Simons et al., 2000; Vinson et al., 2003). These observations prompted us to investigate the possible expression of prominin-1 by these cells. Our data reveal among other things that a specific splice variant of prominin-1 is expressed by oligodendrocytes and appears to be a novel constituent of myelin.

MATERIALS AND METHODS Cell Culture and Transfection

CHO cells were cultured as described previously (Corbeil et al., 2000) and transfected with eukaryotic expression plasmid encoding either prominin-1.s1 or s3 splice variant (Fargeas et al., 2004) using the LipofectAMINE reagent (Gibco BRL, Gaintherburg, MD) according to the supplier's instructions. Cells expressing the neomycin resistance gene were selected in complete medium containing 600 µg/mL of G418. Ten days later, G418-resistant colonies were pooled and expanded in the presence of G418. The cells were incubated for 17 h with 5 mM sodium butyrate to induce the expression of the transgene. Under these conditions, $\approx 20\%$ of the neomycin-resistant cells expressed the recombinant prominin-1.

Primary cultures of oligodendrocytes and astrocytes prepared from embryonic day 14–16 mouse brains were obtained from Jacqueline Trotter (University of Mainz, Germany) and cultured on poly-L-lysine coated glass coverslips for 2–4 weeks as described previously (Trotter and Schachner, 1989).

Cell Extract Preparations

Detergent lysates of prominin-1-transfected CHO cells were prepared as described previously (Corbeil et al., 1999). Detergent lysates derived from primary oligodendrocytes and astrocytes were prepared as described (Krämer et al., 1997). Briefly, primary cells were washed and solubilized directly on the Petri dish with 1% SDS, 20 mM sodium phosphate, pH 7.5. After scraping the cells from the dish, DNA was sheared by several passages through a 22G needle attached to 1-mL syringe. The samples were boiled for 2 min. Protein concentration was determined using BCA Protein Assay Reagent (Pierce, Rockford, IL).

Kidney and Brain Membrane Preparations

Kidneys and brains from adult wild-type NMRI mice or myelin-deficient mutant mice (see below) were homogenized in buffered sucrose (10 mM HEPES-KOH, pH 7.5, 300 mM sucrose, 1 mM EDTA, 1 mM PMSF, 20 μ M leupeptin, 1.5 μ M aprotinin, 1 g tissue/5 mL buffer) at 4°C by 10 strokes in a glass-Teflon homogenizer at 3,000 rpm, and centrifuged at 1,000g for 10 min. The supernatant was centrifuged at 100,000g for 30 min and the resulting membrane pellet was resuspended in 1% SDS, 20 mM sodium phosphate, pH 7.5, and boiled for 2 min. Protein concentration was determined using BCA Protein Assay Reagent.

Three-month-old shiverer $(Mbp^{shi/shi})$ mice originated from homozygous breeding at the animal facility of the University of Heidelberg, and organs from 20-day-old *jimpy* $(Plp^{jp/Y})$ and 28-day-old *rumpshaker* $(Plp^{rsh/Y})$ mutant mice were generously supplied by Klaus Armin Nave (Max-Planck-Institute of Experimental Medicine, Göttingen, Germany). All animals were genotyped before analysis. For *jimpy* and *rumpshaker*, organs of wild-type male littermates were used as controls, whereas for *shiverer*, an age- and sex-matched wild-type mouse was examined. All mutants had the C57BL/6 background.

Myelin Preparation

Myelin was isolated from the brains of adult NMRI mice according to standard procedures (Norton and Poduslo, 1973; Smith, 1969) with minor modifications. Briefly, all solutions were prepared in 10 mM HEPES-KOH, pH 7.5, and a cocktail of protease inhibitors was added. Brains were homogenized in ice-cold 0.3 M sucrose using an Ultra-Turrax T25 (IKA, Staufen, Germany) and centrifuged at 17,000g for 45 min. The upper whitish phase of the pellet (as opposed to the lower reddish blood cell containing phase) was recovered, diluted in 0.85 M sucrose, and subjected to a sucrose step-gradient centrifugation. Myelin was collected from the 0.3 and 0.85 M sucrose interface and subjected to two rounds of hypoosmotic shock by resuspension in a large volume of ice-cold buffered water followed by centrifugation at 68,000g for 30 min. The myelin membranes were further purified on a second sucrose step gradient, washed twice with ice-cold buffered water, pelleted and either resuspended in 1% SDS in water and boiled for 2 min or resuspended in water only. The samples were frozen in small aliquots at -80° C. The protein content was determined using BCA Protein Assay Reagent.

Endoglycosidase Treatment and Immunoblotting

Solubilized kidney and brain membranes $(10-100 \ \mu g \ protein)$ and detergent extracts prepared from transfected CHO cells (one-tenth of a 70% confluent 100-mm dish) were incubated overnight at 37°C in the absence or presence of 1 unit peptide-*N*-glycosidase F (PNGase F) according to the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany).

Proteins were analyzed by SDS-PAGE and transferred to poly(vinylidene difluoride) membranes (Millipore Corp., Beldford, MA; pore size 0.45 μ m) using a semidry transfer cell system (Cti, Idstein, Germany) as described previously (Corbeil et al., 2001a). Membranes were then incubated overnight at 4°C in blocking buffer (PBS containing 5% low fat milk powder and 0.3% Tween 20). Prominin-1 was detected using either the rat mAb 13A4 (1 μ g/mL; Weigmann et al., 1997) or the α I3 antiserum directed against the C-terminal domain of prominin-1.s1 (1:20,000; Corbeil et al., 1999) followed by horseradish peroxidase-conjugated secondary antibodies, all diluted in blocking buffer. Neural cell adhesion molecule L1 was detected using a rabbit polyclonal antibody (1:5,000; Rathjen and Schachner, 1984) (a kind gift of Andreas Faissner, University of Heidelberg), and glial fibrillary acidic protein (GFAP) with a mouse monoclonal antibody (1:300; Boehringer-Manneim, Germany). The antigen-antibody complexes were revealed using horseradish peroxidaseconjugated secondary antibodies followed by enhanced chemiluminescence (ECL system, Amersham Corp.).

Immunofluorescence of Murine Tissues

Cryosections

Brains were dissected from 13-day-old NMRI mouse, embedded in Jung tissue freezing medium, and frozen on powdered dry ice. Cryosections $(12 \ \mu m)$ were cut in a Leica Frigocut 2800N and mounted on TESPA- (3-aminopropyltriethoxysilane, Sigma) coated slides. The sections were air-dried for several hours and fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The fixative was removed by two washes in PBS and reactive groups were quenched in 50 mM NH₄Cl for 10 min. Sections were rinsed twice with PBS, permeabilized with 0.3% saponin in PBS for 15 min, washed three times in PBS, and incubated in blocking buffer (PBS containing 5% FCS and 1% BSA) for 1 h at room temperature. The sections were then double labeled overnight at 4°C with rat mAb 13A4 (10 µg/mL) and rabbit polyclonal antiserum against the C-terminal peptide of myelin proteolipid protein (PLP; 1:500; Linington and Waehneldt, 1990) (a gift of Christopher Linington; Max-Planck-Institute for Neurobiology, Martinsried, Germany) followed by Cy3-conjugated goat anti-rat IgG/IgM (1:150) and Cy2-conjugated goat anti-rabbit IgG/IgM (1:250; Dianova, Hamburg, Germany) for 30 min at room temperature, all diluted in blocking buffer. Unbound antibodies were removed by several washes in PBS, and nuclei were stained by 5-min incubation with Hoechst 33258 (2 µg/mL in PBS; Sigma). The sections were mounted in Mowiol 4.88 (Calbiochem) and viewed with an Olympus BX61 fluorescence microscope.

Lowicryl sections

Various nerves were dissected from an adult NMRI mouse, which had been perfused with 2% formaldehyde, 0.1% glutaraldehyde, 2% poly(vinylpyrrolidone) in 0.1 M PIPES, pH 7.4. They were cut into small pieces, cryoprotected, and frozen in liquid nitrogen. The freeze substitution was performed with methanol containing 1.5% uranyl acetate at -85° C. The samples were infiltrated stepwise with Lowicryl HM20 (Polysciences, Eppelheim, Germany) and polymerized by UV light. Ultrathin sections (\approx 95 nm) were cut, transferred onto a glass coverslip, blocked with 0.8% BSA and 0.1% cold water fish

skin gelatine in PBS, and labeled overnight with rabbit antiserum $\alpha E3$ (1:200) generated against the second extracellular loop of prominin-1 (Maw et al., 2000) or antiserum anti-NCAM (1:100; a gift of Jacqueline Trotter, University of Mainz, Germany) (Trotter et al., 1989) followed by Cy3-conjugated goat anti-rabbit (1:250; Dianova), all diluted in 5% BSA in PBS. Sections were mounted in Mowiol 4.88 and viewed with a ZEISS Axiophot microscope.

Immunofluorescence of Primary Cultures

The primary cells growing on poly-L-lysine coated glass coverslips were washed with PBS, fixed with 4% paraformaldehyde in PBS for 5 min at room temperature, and double labeled with mAb 13A4 (10 μ g/mL) and either mAb anti-O4 (1:50; Sommer and Schachner, 1981), rabbit antiserum against anti-PLP (1:50; Linington and Waehneldt, 1990) or anti-GFAP (1:500; Dakopatts, Hamburg, Germany) followed by the appropriate Cy2/Cy3-conjugated secondary antibody (Dianova) as described (Schnitzer and Schachner, 1981).

Electron Microscopy

Optic nerves, dissected from an 18-day-old C57BL/6 mouse that had been perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, were cut into small pieces, infiltrated overnight with 15% polyvinylpyrrolidone, 1.95 M sucrose in PBS (Tokuyasu, 1989), and frozen in liquid nitrogen. Ultrathin cryosections (Griffiths, 1993) were prepared using a Reichert FCS cryo-ultramicrotome (Leica, Vienna, Austria) and collected on Formvar carbon-coated grids. Sections were blocked with 10% FCS and 20 mM glycine in PBS for 20 min, and sequentially incubated with mAb 13A4, rabbit anti-rat IgG/IgM antibody (Jackson ImmunoResearch Laboratories), and protein A coupled to 9 nm gold, each diluted in blocking solution containing only 5% FCS. After several washing steps with the blocking solution and PBS, sections were postfixed with 1% glutaraldehyde in PBS for 5 min, washed extensively in distilled water, and treated with 0.3% uranyl acetate/1.8% methylcellulose, and air-dried as described previously (Corbeil et al., 1999). The immunogold-labeled sections were examined by electron microscopy (Zeiss EM 10; LEO, Oberkochen, Germany).

Combined In Situ Hybridization and Immunohistochemistry

Tissue samples

Ten-day-old and adult mouse brain samples were obtained from C57BL/6 strain. Mice were deeply anesthetized by a single intraperitoneal bolus injection of Ketamine and Xylazin mixture. Animals were then transcardially perfused with ice-cold 4% paraformaldehyde. The brain was removed and postfixed in 4% paraformaldehyde for 2 h at 4°C. After cryoprotection with 30% sucrose-PBS, tissue samples were embedded in OCT compound (Tissue Tek, Sakura, The Netherlands). Samples were sectioned on a cryostat (HM560, Microm International GmbH, Walldorf, Germany) at 12 μ m and then mounted onto SuperFrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany), dried overnight at room temperature, and stored at -20° C until use.

cRNA probe

Antisense complementary DIG-labeled ribonucleic acid (cRNA) probe was generated using T7 RNA polymerase and DIG labeling mix (Roche Molecular Biochemicals). To synthesize murine Prominin-1 (Accession number AF026269) cRNA probe a 2.1 kb (nt 198-2264) cDNA fragment was used.

In situ hybridization and immunohistochemistry

Nonradioactive in situ hybridization (ISH) on 4% paraformaldehyde-fixed mouse cryosections was performed according to standard protocols (Tiveron et al., 1996). Briefly, serial sections were hybridized with digoxygenin (DIG) labeled cRNA probe (see above) at a concentration of 0.5 ng/µL for 16 h at 70°C. Stringency washes were performed at 70°C. The sections were then incubated with anti-DIG antibody (1:4,000; Roche Molecular Biochemicals) for 16 h at 4°C. After several washing steps, the reaction was visualized using NBT-BCIP substrate (Roche Molecular Biochemicals) giving a blue reaction product. After stopping the color reaction by several washes in PBS, the sections were further processed for Olig-2 immunohistochemistry. Sections were blocked with 10% horse serum (HS) in 0.2% Triton X-100 in PBS (PBSTx) for 1 h and then incubated with rabbit anti-Olig-2 antiserum (1:1,000; Chemicon) overnight at 4°C. The samples were extensively washed with PBSTx followed by an additional incubation in 10% HS PBSTx for 30 min. The primary antibody was detected with a biotinylated goat anti-rabbit secondary antibody (1:500; Vector Laboratories, Burlingame, CA), avidin-biotin-peroxidase complex (ABC Elite Vectastain kit, Vector Laboratories), and DAB chromogen. After washing once with PBS, the slides were rinsed quickly in dH₂O and mounted with Kaiser's Glycerol-Gelatin (Merck, Darmstadt, Germany). Images were captured using an Olympus BX61 compound microscope with the IPLAB software. The composite images were prepared from the digital data files using Adobe Photoshop and Illustrator.

RESULTS Expression and Characterization of Prominin-1 in Adult Mouse Brain

Immunoblotting of adult mouse kidney and brain membrane lysates using mAb 13A4 (Weigmann et al., 864



Expression of the prominin-1.s3 variant in adult mouse Fig. 1. brain. (A) The brain-associated form of prominin-1 is distinct from the kidney-derived prominin-1 splice variant. Proteins solubilized from adult mouse kidney (10 µg) and brain membranes (40 µg) were analyzed by immunoblotting using either mAb 13A4 (left panel) or aI3 antiserum directed against the C-terminal domain of prominin-1.s1 (right panel). Arrows, 115-kDa form of kidney prominin-1; arrowheads, 100-kDa form of brain prominin-1. (B) C-terminal protein sequences of prominin-1.s1 and s3 variants. Color code refers to distinct facultative exons. (C) Brain-associated prominin-1 and recombinant prominin-1.s3 variant exhibit a similar electrophoretic mobility. Proteins solubilized from adult mouse brain membranes (100 $\mu g)$ and CHO cells stably transfected with prominin-1.s1 or s3 variants were incubated in the presence of 1 unit PNGase F and analyzed by immunoblotting using mAb 13A4. Open arrow, N-deglycosylated, 94-kDa form of recombinant prominin-1.s1; open arrowhead, N-deglycosylated, 90-kDa form of brain prominin-1 and of recombinant prominin-1.s3.

1997), which recognizes an epitope located in the second extracellular loop of prominin-1 (Maw et al., 2000), demonstrated that the brain-associated prominin-1 has a faster electrophoretic mobility with an apparent molecular mass of 100 kDa (Fig. 1A, left panel, arrowhead), than the 115-kDa prominin-1 found in kidney (Fig. 1A, left panel, arrow). The same data were obtained using a rabbit aE2 antiserum generated against the first extracellular loop of prominin-1 (Corbeil et al., 1999) (data not shown). Interestingly, brain-associated prominin-1 did not show any immunoreactivity (Fig. 1A, right panel, arrowhead) when using the rabbit α I3 antiserum (Corbeil et al., 1999) specific for the cytoplasmic C-terminal domain of the prominin-1.s1 splice variant (GenBank accession number AF026269), which is expressed in kidney (Fig. 1A, right panel, arrow), suggesting either a brain-specific proteolytic digestion of prominin-1 or the expression of a distinct splice variant. Indeed, several prominin-1 splice variants with different cytoplasmic Cterminal domains as compared to the kidney-derived prominin-1.s1 have been isolated and characterized previously (Fargeas et al., 2004) (for the nomenclature of prominin-1 variants see Fargeas et al., 2007). One of these variants, which results from the use of an alternative splice acceptor and is referred to as s3 (GenBank accession number AF305215), exhibits a smaller cytoplasmic C-terminal tail (Fig. 1B; Fargeas et al., 2004).

We therefore compared the electrophoretic mobility of brain-associated prominin-1 with recombinant prominin-1.s1 and s3 variants expressed in CHO cells. Upon removal of N-linked glycans by PNGase F treatment,



Fig. 2. Developmental expression of prominin-1 splice variants in mouse brain. Proteins solubilized from adult mouse kidney membranes (10 µg) and mouse brain membranes (40 µg) at different developmental stages were incubated in the absence (top panel) or presence (middle and bottom panel) of 1 unit PNGase F and analyzed by immunoblotting using either mAb 13A4 (top and middle panel) or α I3 antiserum (bottom panel). Arrow, 115-kDa form of kidney prominin-1.s1 variant; arrowhead, 100-kDa form of brain prominin-1.s3 variant; open arrows, N-deglycosylated, 94-kDa form of kidney prominin-1.s1; open arrowheads, N-deglycosylated, 90-kDa form of brain prominin-1.s3. E, embryonic day; P, postnatal day.

prominin-1 found in the brain and recombinant prominin-1.s3 showed a very similar electrophoretic migration, with an apparent molecular mass of 90 kDa (Fig. 1C, arrowhead). Recombinant prominin-1.s1 displayed a higher molecular mass, that is, 94 kDa, (Fig. 1C, arrow), like kidney-derived prominin-1 (see below, Fig. 2). Moreover, prominin-1.s3 mRNA was detected in mouse brain by PCR amplification using a set of specific oligonucleotide primers (data not shown). Taken together, these results show that the prominin-1 splice variant expressed in adult mouse brain is s3.

Expression of Prominin-1 Splice Variants in Mouse Brain Is Developmentally Regulated

We examined the expression of prominin-1.s1 and s3 variants during the development of the mouse brain. We previously reported that the s1 variant is expressed in embryonic mouse brain, specifically, in neuroepithelial cells (Weigmann et al., 1997). At an early stage, that is,



Fig. 3. Prominin-1 transcript is expressed in Olig-2-positive oligodendroglial cells of postnatal mouse brain. Cryosections of a 10-day-old mouse brain from the forebrain (A-D) and hindbrain (E) were processed for nonradioactive ISH (*prominin-1*; blue) followed by immunohistochemistry (Olig-2; brown). (A-E) *Prominin-1*/Olig-2 double-positive oligodendroglial cells (solid arrowheads) are concentrated within white matter tracts (dashed lines) in the forebrain (A-D) and distributed in a

scattered manner throughout the hindbrain (E). Not all Olig-2-positive cells expressed *prominin-1* transcript (open arrowheads). The insets in panels B, D, and E demarcate a region displayed at higher magnification in **b**, **d**, and **e**, respectively. CC, *corpus callosum*; CI; *capsula interna*; FR, *formatio reticularis*; 3V, third ventricle. Bars, 50 μ m (A, b, C, d, e) and 100 μ m (B, D, E).

in the 10-day-old embryo (E10), two distinct 13A4-immunoreactive bands were observed (Fig. 2, top panel). At later embryonic stages (E13 to E16), the 13A4-immunoreactivity appeared as a broad band with an apparent molecular mass of $\approx 105-112$ kDa, which was downregulated during the early postnatal days (P0 to P6). Subsequently, the apparent molecular mass of prominin-1 decreased further, while its abundance increased. At



Fig. 4. Prominin-1 transcript is expressed in Olig-2-positive oligodendroglial cells of adult mouse brain. Cryosections of a 3-month-old mouse brain from the forebrain (**A–D**) were processed for nonradioactive ISH (prominin-1; blue) followed by immunohistochemistry (Olig-2; brown). Only a subpopulation of Olig-2-positive oligodendroglial cells express strongly the prominin-1 transcript (solid arrowheads). Olig-2-

P16 and thereafter, prominin-1 appeared as a distinct band of 100 kDa, identical to the one observed in adult brain (Fig. 2, top panel, arrowhead), suggesting that expression of the s1 variant is downregulated during brain development whereas the opposite is the case for the s3 variant. To address this issue, samples were incubated with PNGase F and deglycosylated proteins analyzed by immunoblotting using either the mAb 13A4 or αI3 antiserum. As expected, the 94-kDa deglycosylated s1 variant, which is recognized by both antibodies, was downregulated during brain development and almost undetectable in adult brain (Fig. 2, middle and bottom panels, arrow). Conversely, the 90-kDa deglycosylated s3 variant, which is recognized by mAb 13A4 (Fig. 2, middle panel, arrowhead), but not the α I3 antiserum (Fig. 2, bottom panel, arrowhead), was upregulated and eventually constituted the major prominin-1 splice variant expressed in adult mouse brain (Fig. 2, middle

positive/prominin-1 transcript-negative cells (open arrowheads). The insets in panels A, and B demarcate a region displayed at higher magnification in **a** and **b**, respectively. CC, corpus callosum; Fx, fornix; ST, stria terminalis; CA, commissural anterior; 3V, third ventricle. Bars, 100 μ m (A, B) and 50 μ m (a, b, C, D).

panel, arrowhead). Notably, the steep increase in prominin-1.s3 expression in postnatal stages coincided with the beginning of the myelination period, 2 weeks after birth.

Prominin-1 Transcript Is Detected in a Subpopulation of Olig-2-Positive Cells

Our biochemical observations prompted us to investigate the expression of prominin-1 relative to the oligodendrocyte lineage in the postnatal 10-day-old mouse brain. An approach that combined both ISH and immunohistochemistry was applied. We used Olig-2, a bHLH transcription factor, as a marker of the oligodendroglial lineage including mature oligodendrocytes (Lu et al., 2000; Zhou et al., 2000). Interestingly, *prominin-1* transcripts were detected in cell populations located almost



Fig. 5. Prominin-1 protein is expressed in cerebellar white matter. Sagittal cryosections of a 13-day-old mouse brain were double labeled with mAb 13A4 (anti-prominin-1; left top panel) and anti-proteolipid protein (PLP; right top panel), and analyzed by immunofluorescence

exclusively in major white matter tracts of the forebrain (Fig. 3A-D, blue cells). These expression sites included among others the corpus callosum (Fig. 3A-C,b), the capsula interna (Fig. 3D,d), and the fornix (data not shown). All prominin-1-positive cells co-expressed Olig-2 (Fig. 3A-C,b, brown nuclei, arrowheads). These doublepositive cells nevertheless accounted only for a fraction of the total Olig-2-positive cell population (Fig. 3A-D,b). Outside the white matter tracts, prominin-1-positive cells were scarcely found (Fig. 3A–D,b; data not shown). In contrast, the hindbrain reticular formation harbored a more scattered pool of prominin-1/Olig-2 expressing cells (Fig. 3E,e, arrowheads). Again, these cell populations represented only a fraction of the total Olig-2-positive cells. Beyond the reticular formation, the superficial hindbrain white matter tracts and the cerebellar pedunculi upon entering the cerebellum (corpus medullare cerebelli) were also enriched in prominin-1/Olig-2 positive cells (data not shown).

It is worth mentioning that cortical neurons composing the telecephalic commissural systems and major descending pathway were negative for *prominin-1* transcript (data not shown). Similarly, neuronal populations, that is, discrete anatomical nuclei, (e.g., pontine nuclei, *oliva inferior*, *nucl. vestibularis lateralis*, Purkinje cells/ cerebellar nuclei), whose projective fibers compose the major afferent or efferent tracts of the cerebellum appeared to be negative for *prominin-1* transcript (data not shown).

microscopy. Nuclei were labeled with Hoechst dye (right bottom panel). As a negative control, anti-prominin-1 antibody was omitted (left bottom panel). WM, white matter; GL, granule cell layer; ML, molecular layer. Bar, 100 μ m.

Because the *prominin-1*/Olig-2 expressing cells observed in the postnatal brain might be related to the late progenitors, we have investigated the expression of the *prominin-1* transcript in the adult brain as well. Again we could detect such *prominin-1*/Olig-2 doublepositive cells in 3-month-old animal, although their frequency appeared reduced in comparison to the postnatal brain (see Fig. 4). Interestingly, Jalabi et al. (2003) have previously observed a drastic downregulation of messages encoding for two myelin-associated proteins, that is, PLP and myelin oligodendrocyte glycoprotein in older animals suggesting that not all mature glial cells display a transcriptional activity in a given time.

Prominin-1 Is Localized to the Myelin Sheath of Central and Peripheral Nerves

Because the absence of prominin-1 transcripts in certain Olig-2-positive cells does not rule out the presence of its product and, given that Olig-2-positive cells have the capacity to develop into various lineages as suggested by recent lineage tracing experiments (Ono et al., 2008), we decide to investigate the actual expression of prominin-1 protein in mouse brain by immunohistochemistry using either mAb 13A4 or the rabbit α E3 antiserum. Labeling of the cerebellar cortex of a postnatal 13-day-old mouse revealed the presence of prominin-1 in white matter (Fig. 5, top left panel, WM), which



Fig. 6. Prominin-1 protein is expressed in central and peripheral nerves of adult mouse. Lowicryl sections of different nerves from adult mice were incubated with $\alpha E3$ antiserum against prominin-1 (upper left and bottom panels) or NCAM antiserum (upper right panel). Top panels; prominin-1 is detected in myelin sheaths of the optic nerve

was identified by staining for PLP (Fig. 5, top right panel), the major constituent of myelin. No significant prominin-1 immunoreactivity was observed in the granule cell layer (GL), Purkinje cells, and molecular layer (ML) (Fig. 5, top left panel) consistent with ISH (Figs. 3 and 4), or when mAb 13A4 was omitted (Fig. 5, bottom left panel). In adult mice, prominin-1 immunoreactivity is detected in the optic nerve (Fig. 6, top left panel, ON) and the neighboring peripheral cranial nerves ensheathed by neural crest-derived Schwann cells (Fig. 6, top left panel, PN). For comparison, an adjacent section was stained with an antibody against the neural cell adhesion molecule (NCAM) (Fig. 6, top right panel) (Bhat and Silberberg, 1988). Likewise, sciatic and trigeminal nerves displayed prominin-1 immunoreactivity (Fig. 6, bottom left and right panels, respectively). In these cases, prominin-1 immunoreactivity appeared as a ring suggesting that prominin-1 is associated with the myelin sheath. Finally, the presence of prominin-1 immunoreactivity in myelin was investigated by immunogold electron microscopy. Labeling of the optic nerve with mAb 13A4 revealed the association of prominin-1 immunoreactivity with compacted lamellae of myelin

(ON) and adjacent peripheral nerves (PN) (left panel), like NCAM (right panel). Bottom panels; prominin-1 is detected in the myelin sheath of the sciatic nerve (left panel) and the trigeminal nerve (right panel). Bars, 10 μ m (left bottom panel) and 16 μ m (right panels).

(Fig. 7, arrowheads, MS). No gold particle was observed in the axons ensheathed by the myelin (Fig. 7, Ax) in agreement with the ISH data (see above).

Prominin-1 Is Found in the Myelin Fraction of Adult Mouse Brain

To corroborate our morphological data, we investigated the association of prominin-1 with myelin by a biochemical approach. A whole brain homogenate was fractionated and myelin enriched by sucrose density gradient centrifugation (Norton and Poduslo, 1973). To monitor the enrichment of myelin proteins, an aliquot of each fraction was analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 8, prominin-1 was enriched in the myelin fraction (Fig. 8, top panel, arrowhead), whereas the neural cell adhesion molecule L1, a neuronal marker, was significantly reduced (Fig. 8, middle panel, arrow). The validity of subcellular fractionation was confirmed by the pattern of proteins, showing the enrichment of PLP (30 kDa) and myelin basic protein (MBP) isoforms (14 and 21 kDa) (Fig. 8, bottom panel,



Fig. 7. Immunogold electron microscopy reveals presence of prominin-1 in optic nerve myelin sheath. Ultrathin cryosections of mouse optic nerve were stained with mAb 13A4 (anti prominin-1) followed by rabbit anti-rat secondary antibody and 9-nm Protein A-gold. Arrowheads indicate prominin-1 immunoreactivity in the myelin sheath. Ax, axon; MS, myelin sheath. Bar, 230 nm.

open and closed diamonds, respectively) and the decrease of other proteins (Fig. 8, bottom panel, circles) in the myelin fraction. Finally, the PNGase F-treated Ndeglycosylated prominin-1 enriched in the myelin fraction showed the same electrophoretic migration as the prominin-1.s3 variant found in adult brain, with an apparent molecular weight of 90 kDa (data not shown), suggesting that the s3 variant of prominin-1 is expressed by oligodendrocytes in the CNS.

Prominin-1 Is Reduced in Brain of Myelin-Deficient Mice

The finding that prominin-1.s3 appears to be a constituent of myelin prompted us to assess its expression in dysmyelinating disorders. Interestingly, the expression of prominin-1, while normal in the kidney (Fig. 9, shiverer, arrow), was below the detection level in the brain of dysmyelinated shiverer mice (Fig. 9, shiverer, arrowhead), an autosomal recessive deletion mutant of the *mbp* gene (Roach et al., 1985) that is characterized by a lack of all isoforms of MBP resulting in the formation of little or no compact myelin in the CNS (Chernoff, 1981). A similar situation was observed in mice carrying the spontaneous plp mutation jimpy (Fig. 9, jimpy, arrowhead), which provokes a lethal disorder with many apoptotic oligodendrocytes and severe hypomyelination (Knapp et al., 1986; Nave et al., 1986). Remarkably, the expression of prominin-1 was less severely reduced compared to wild type in the brain of mice bearing the rumpshaker mutation (Fig. 9, rumpshaker, arrowhead), an allele of *plp*, that confers a milder condition, with a



Fig. 8. Prominin-1 is enriched in the myelin fraction from adult mouse brain. A homogenate of adult mouse brain was fractionated into a 17,000g pellet and supernatant. The upper phase of the pellet was collected and subjected to a first sucrose step gradient. The myelinenriched interface was collected and subjected to a second sucrose step gradient. The resulting interface is referred to as myelin fraction. (The pellet of the first step gradient was analyzed in parallel). Proteins (80 µg) of the different fractions were analyzed by SDS-PAGE followed by immunoblotting using mAb 13A4 (anti prominin-1) (top panel) and L1 (middle panel) or Coomassie Blue staining (C.B., bottom panel). Arrowhead, prominin-1; arrow, L1. Diamonds and circles indicate proteins that are enriched or depleted during myelin purification, respectively. Open diamond, PLP; close diamonds, MBP.

normal number of mature oligodendrocytes and more myelin (Griffiths et al., 1990; Schneider et al., 1992).

Prominin-1 Is Expressed in Glial Cells Cultured In Vitro

We next investigated the expression of prominin-1 in glial cells cultured *in vitro*. Immunoblotting showed a



Fig. 9. Prominin-1 is reduced in the brain of myelin-deficient mice. Proteins of brain membranes of three myelin-deficient mouse mutants (mut), shiverer (40 μ g), jimpy (40 μ g), and rumpshaker mice (50 μ g), or wild-type (wt) (40 or 50 μ g) mice were analyzed by immunoblotting using mAb 13A4 (anti prominin-1). For comparison, proteins from kidney membranes (10–20 μ g) of the same individual animals were analyzed in parallel. Equal amounts of proteins were loaded for the mutants and wild-type animals. Arrows, 115-kDa form of kidney prominin-1.s1; arrowheads, 100-kDa form of brain prominin-1.s3.

single band with the same electrophoretic mobility as the myelin-associated prominin-1.s3 variant in lysates of primary oligodendrocytes after 3 days of culture (Fig. 10A, top panel, arrowhead). Upon longer culture, for example, 10 days, a second prominin-1 immunoreactive band appeared with an electrophoretic mobility corresponding to that of the prominin-1.s1 variant found in kidney (Fig. 10A, top panel, arrow). However, after 10 days, in vitro cultures of primary oligodendrocytes are known to contain astrocytes (Niehaus et al., 1999) as confirmed by the presence of immunoreactivity for GFAP (Reeves et al., 1989), a specific astrocyte marker (Fig. 10A, bottom panel). Given that primary astrocytes express the prominin-1.s1 variant (Fig. 10A, top panel), the presence of s1 variant in the 10-day-old oligodendrocyte cultures therefore likely reflects the presence of astrocytes.

Expression of prominin-1 in glial cells was further investigated by immunofluorescence on 2- and 4-weekold cultures. Both developing and well-differentiated oligodendrocytes, which were labeled for the O4 antigen (Sommer and Schachner, 1981) and PLP, respectively, displayed prominin-1 immunoreactivity (Fig. 10B). Likewise, astrocytes present in the 4-week-old culture also contained prominin-1 immunoreactivity, which appeared concentrated in the leading edge (Fig. 10B, arrows). Not all GFAP-positive cells expressed prominin-1 (Fig. 10B, asterisk).

DISCUSSION

In this study, we report three important observations. First, different splice variants of prominin-1 with distinct cytoplasmic C-terminal domains are developmentally regulated in mouse brain. Second, the expression of the s3 splice variant follows the maturation of oligodendrocytes and myelination, whereas the relative abundance of the s1 splice variant, which is expressed in neuroepithelial progenitor cells in early embryos, decreased toward adulthood. Third, the s1 and s3 splice variants are expressed by primary astrocytes and oligodendrocytes, respectively.

The presence of prominin-1 in myelin is interesting given that the myelin membrane contains a specific lipid composition being highly enriched in cholesterol and two glycosphingolipids, galactocerebroside and its sulfated derivative galactosulphocerebroside (Lee, 2001). Keeping in mind the postulated function of prominin-1, that is, organizing plasma membrane protrusions (Corbeil et al., 2001b), prominin-1 may supply the plasma membrane outgrowths of glial cells with an appropriate lipid composition, notably with respect to plasma membrane cholesterol, an interaction partner of prominin-1 (Röper et al., 2000). Alternatively, prominin-1 may represent a new class of adhesion molecules, with its two large glycosylated extracellular loops engaged in either homo- or heterophilic interactions (Jászai et al., 2007b), and hence may play a certain role in myelin compaction and/ or axon ensheathment. In both scenarios, which are not mutually exclusive, the information presented here will help to understand the molecular organization of the myelin sheath. The characteristic organization of myelin is essential for the function of the nervous system, and any alteration of its architecture leads to severe neurological diseases.

Is prominin-1 essential for the complex process of myelination? Individuals carrying a single nucleotide deletion (nt 1878) in the human PROMININ-1 gene, which results in a frameshift at codon 614 with premature termination of translation, exhibit no other symptoms than a progressive retinal degeneration (Maw et al., 2000), which suggests that prominin-1 is dispensable for myelination. However, the absence of an evident phenotype in these patients does not rule out that prominin-1 exerts a certain role in myelin biogenesis and/or its maintenance given the propensity of prominin-1 to undergo alternative splicing (Fargeas et al., 2004, 2007). Specifically, the exon carrying the single-nucleotide deletion could be skipped, resulting in a shorter extracellular loop but maintaining the pentaspan structure of the molecule in tissues other than retina. The expression of the prominin-1.s3 variant in both developing and more mature oligodendrocytes cultured in vitro broadens the spectrum



Fig. 10. Prominin-1 splice variants are differentially expressed in glial cells in vitro. (A) Cultured oligodendrocytes express prominin-1.s3 while astrocytes express the s1 variant. Proteins from adult mouse kidney membranes (10 μ g), myelin (40 μ g), homogenates of primary oligodendrocytes cultured for 3 and 10 days (3 d, 10 d) (100 μ g), and primary astrocytes (20 μ g) were analyzed by SDS-PAGE followed by immunoblotting using either mAb 13A4 (anti prominin-1, top panel) or GFAP antiserum (bottom panel). Note that the expression of the prominin-1.s1 variant in the 10-day oligodendrocyte culture is likely due to the presence of astrocytes. Arrow, 115-kDa form of kidney prominin

1.s1; arrowhead, 100-kDa form of brain prominin-1.s3. (**B**) Expression of prominin-1 in primary oligodendrocytes and astrocytes as revealed by immunofluorescence microscopy. Oligodendrocytes cultured for 2 (left column) or 4 weeks (middle and right column) on poly-t-lysine coated glass coverslips were double immunolabeled with mAb 13A4 (anti-prominin-1) and either anti-O4 (left column) or anti-PLP antibody (middle column). Astrocytes present in the 4-week-old culture were double immunolabeled with mAb 13A4 and anti-GFAP antibody (right column). Arrows, prominin-1 staining at the leading edge; asterisk, astrocyte that does not express prominin-1. Bar, 20 μ m.

of approaches that can be taken to investigate its precise role in myelination. Therefore, further studies addressing the spatio-temporal expression of these prominin-1 splice variants in primary cultured cells are necessary. In addition to its early expression in neuroepithelial progenitor cells that give rise to all neurons and macroglial cells of the CNS (Weigmann et al., 1997), neural stem cells from postnatal cerebellum (Lee et al., 2005), and adult ependymal cells (Coskun et al., 2008; Huttner et al., 2008; Pfenninger et al., 2007; Weigmann et al., 1997), prominin-1 is found to be expressed in glial cells. The detection of the prominin-1.s1 variant in a subpopulation of cultured GFAP-positive astrocytes (see Fig. 10) is highly interesting in light of recent publications revealing the heterogeneity of astrocytes (Ruiz-Ederra et al., 2003; Scotti Campos, 2003). The existence of distinct astrocyte populations particularly with regard to the stem cell marker prominin-1 is a crucial issue, given that astrocytes may function as stem cells in adult mammalian brain (Doetsch et al., 1999). Thus, prominin-1 could be a useful tool to isolate and characterize further different populations of astrocytes and those that belong to stem cell lineages (Alvarez-Buylla et al., 2002; Götz et al., 2002). Indeed, some preliminary observations are going in this direction (Tripathi and Götz, 2007).

Finally, it is interesting to note the last four C-terminal amino acid stretch of the s3 splice variant, that is, HFTL, exhibits the characteristics of a class II PDZ binding domain with a hydrophobic residue in position 0 and -2 (X- Φ -X- Φ ; X, unspecified amino acid; Φ , hydrophobic residue) (Harris and Lim, 2001; Songyang et al., 1997), whereas the C-terminal end of the s1 splice variant, that is, PSRY, is highly related to a class I PDZ binding domain (X-S/T-X-V/L) (Fargeas et al., 2007). Whether prominin-1 molecules interact with various PDZ domain-containing proteins still needs to be assessed, yet the presence of a distinct C-terminal domain in the s3 splice variant suggests strongly that myelin-associated prominin-1 might interact with an alternative cytoplasmic protein partner. Further studies are clearly necessary to determine the physiological relevance of such putative interaction, in particular in the context of myelination.

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