OSP/Claudin-11 Forms a Complex with a Novel Member of the Tetraspanin Super Family and β1 Integrin and Regulates Proliferation and Migration of Oligodendrocytes

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Abstract. Oligodendrocyte-specific protein (OSP)/claudin-11 is a major component of central nervous system myelin and forms tight junctions (TJs) within myelin sheaths. TJs are essential for forming a paracellular barrier and have been implicated in the regulation of growth and differentiation via signal transduction pathways. We have identified an OSP/claudin-11–associated protein (OAP)1, using a yeast two-hybrid screen. OAP-1 is a novel member of the tetraspanin superfamily, and it is widely expressed in several cell types, including oligodendrocytes. OAP-1, OSP/claudin-11, and β1 integrin form a complex as indicated by coimmunoprecipitation and confocal immunocytochemistry. Overexpression of OSP/claudin-11 or OAP-1 induced proliferation in an oligodendrocyte cell line. Anti–OAP-1, anti–OSP/claudin-11, and anti–β1 integrin antibodies inhibited migration of primary oligodendrocytes, and migration was impaired in OSP/claudin-11–deficient primary oligodendrocytes. These data suggest a role for OSP/claudin-11, OAP-1, and β1 integrin complex in regulating proliferation and migration of oligodendrocytes, a process essential for normal myelination and repair.

Key words: tight junctions • myelin • TM4SF • brain • OAP-1

Introduction

Myelin is essential for the effective and rapid propagation of action potentials and therefore, the functional integrity of the nervous system. Membrane processes from oligodendrocytes in the central nervous system (CNS)1 and Schwann cells in the peripheral nervous system wrap around axons in a tight spiral-like manner, increasing the resistance to current perpendicular to the axons. Oligodendrocytes arise from precursor cells located in ventral regions of the neural tube during development of the CNS. These precursor cells must proliferate, migrate throughout the CNS, and differentiate in order to form mature myelin. The molecular mechanisms modulating these events are not completely understood but can be directed in culture by several growth factors, including platelet-derived growth factor and basic FGF (Levine and Dou, 1991). In recent years, there has been considerable progress in the identification of glial proteins involved in the elaboration and maintenance of myelin. Myelin basic proteins (MBPs) and proteolipid protein (PLP) make up nearly 80% of the total myelin proteins in the CNS (for reviews see Campagnoni, 1988; Bronstein et al., 1997) with myelin–oligodendrocyte protein (Gardinier et al., 1992), myelin–oligodendrocyte basic protein (Holz et al., 1996), and myelin-associated glycoprotein contributing as minor components (for review see Campagnoni and Macklin, 1988).

Oligodendrocyte-specific protein (OSP) is a putative four-transmembrane protein that is primarily expressed in oligodendrocytes of the CNS and Sertoli cells of testes in the adult mouse (Bronstein et al., 1996; Morita et al., 1999). OSP is the third most abundant CNS myelin protein, contributing 7% of the total myelin protein (Bronstein et al., 1997), and was found recently to share sequence homology with the claudin family of tight junction (TJ) proteins (Morita et al., 1999). OSP forms TJs in cell culture and to more accurately reflect its function, OSP was renamed OSP/claudin-11 (Morita et al., 1999). Although classical TJs have not been described in myelin, similar structures have been reported (Dermietzel et al., 1980), and OSP/claudin-11 localizes to these structures (Morita et al.,...
Three OSP/claudin-11 constructs were used as bait for yeast two-hybrid screen: the entire OSP/claudin-11 ORF, the first extracellular domain of OSP/claudin-11 between 35 and 84 amino acids, and the COOH terminus of OSP/claudin-11 between 122 and 207 amino acids. These cDNAs were cloned into the pGBT9 backbone (Matchmaker system; CLONTECH Laboratories, Inc.). GAL4 activation domain cDNA fusion libraries were constructed in modified pGAD GH vector (CLONTECH, Laboratories, Inc.) using mRNA from mouse brain. Plasmids were transfected into JM101-competent bacterial cells and amplified once on agar plates.

### Materials and Methods

#### Yeast Two-Hybrid Bait and Library Constructions

Three OSP/claudin-11 constructs were used as bait for yeast two-hybrid screen: the entire OSP/claudin-11 ORF, the first extracellular domain of OSP/claudin-11 and the COOH terminus of OSP/claudin-11. These cDNAs were cloned into the pGBT9 backbone (Matchmaker system; CLONTECH Laboratories, Inc.). GAL4 activation domain cDNA fusion libraries were constructed in modified pGAD GH vector (CLONTECH, Laboratories, Inc.) using mRNA from mouse brain. Plasmids were transfected into JM101-competent bacterial cells and amplified once on agar plates.
centration of 10^7 cpn/ml of hybridization solution. After hybridization, hydrolysis of nonspecifically hybridized probe was initiated by treatment of brain sections with 30 μg/ml ribonuclease A in 10 mM Tris saline with 1 mM EDTA for 40 min at 45°C. Subsequently, the sections were rinsed through descending concentrations of SSC buffer containing 100 mM sodium thiocyanate. The distribution of hybridization was localized by an exposure to Beta Max film (Amersham Pharmacia Biotech) and Eastman Kodak Co. NTB2 emulsion. Films were exposed for 2-4 d and subjected to liquid emulsion (Ilford L-48). Tissue sections were counterstained with hematoxylin and eosin after autoradiographic development.

**Cell Culture**

**Cell Line.** A conditionally immortalized mouse oligodendrocyte cell line (CIMO) (Bronstein et al., 1998) was grown in DME/HAM F12 supplemented with 5% FCS, transferrin, selenium (Reduser, Upstate Biotechnology), and gamma interferon (1; 100 U/ml) (GIBCO BRL) at 33°C or 37°C in 5% CO₂. Cells were either maintained in medium with 1 ml at 33°C (permissive) or 37°C in medium lacking 1 ml (nonpermissive). CIMO cells were stable-transfected with pBabe vector, OSP/claudin-11, antisenese OSP/claudin-11, PLP, and MBP in pBabe vector separately and pcDNA3.1 vector alone and OAP-1 in pcDNA3.1 vector. DOTAP liposomal transfection reagent (Boehringer) was used for transfection according to the manufacturer’s protocol. Stable transformants were maintained after antibiotic (neomycin 400 μg/ml for pBabe vector background and genticin 500 μg/ml for pcDNA vector background) selection.

**Purification of Oligodendrocytes.** Purified oligodendrocytes were obtained by a technique modified from McCarthy and de Vellis (1980). In brief, primary cultures were established from rat and mice (wild-type, OSP/claudin-11 homozygous, and heterozygote knockout) neonatal forebrain cells obtained by dissociating cerebral cortices in papain and grown for ~10 d in DME/F12 supplemented with 10% heat-inactivated FCS. The flasks were shaken overnight to separate the loosely attached oligodendrocyte precursors. These cells were centrifuged and resuspended in appropriate medium and used for immunocytochemistry and cell migration assays (Milner et al., 1996).

**Antibodies**

Anti-OSP/claudin-11 antibody was made as described previously (Bronstein et al., 1997). Rabbit polyclonal antibodies for OAP-1 were raised against an 18-amino acid peptide (H-YSDWNTDFWKTENKOSV-OH) conjugated to keyhole lumpect hemocyanin (Research Genetics). The 18 amino acids (153–170) (see Fig. 1) correspond to a region within the putative second extracellular loop of OAP-1. Antibody specificity was confirmed by Western blot, immunohistochemistry, and peptide competition experiments. Anti-GFAP antibody, peroxidase-conjugated, and horse-radish-conjugated goat anti-rabbit antibody were purchased from Zymed Laboratories. Anti-β1 integrin antibody (Chemicon), Texas red-labeled and FITC-labeled goat anti-rabbit antibody, FITC-labeled goat anti-mouse antibody (Vector Laboratories), and anti-GalC antibody (Boehringer) were used in different concentrations for immunohistochemistry and in some cases for Western blots and migration assays.

**Immunohistochemistry and Confocal Microscopy**

Oligodendrocytes were grown on Lab-Tek chamber slides for 4–8 d. For experiments in fixed cells, cultures were washed with PBS, fixed with 4% paraformaldehyde for 20 min at 4°C, and treated with 0.3% TX-100 in PBS plus 2% normal goat serum for 10 min at room temperature either before or after incubation of cells with primary antibodies. Some cells were also treated with 100% ice-cold methanol for 10 min at −20°C. Both live and fixed cells were blocked for 2 h in 25% normal goat serum in TBS; primary antibody was either added for 2 h at room temperature or overnight at 4°C. Antibody dilutions were as follows: OSP/claudin-11, 1:50; OAP-1, 1:250; and β1 integrin, 1:50. Antigen–antibody interactions were visualized with anti-rabbit IgG conjugated to Texas red (13,000) and FITC (12,000) and anti-mouse IgG conjugated to FITC (1:1,000). Live cells were then fixed with 4% paraformaldehyde. After staining, slide chambers were removed, and slides were coveredslipped with Fluoromount (Fisher Scientific).

Cells were examined with a ZEISS LSM 410 laser confocal microscope system. The excitation source was a krypton argon laser (Coherent) with output at 488, 568, and 633 nm. Fluorescein fluorescence was imaged with a 488-nm emission filter and a 515–540-nm band pass filter. Texas red fluorescence was imaged with a 568-nm emission filter. The resulting images were created by projecting several optical sections obtained at different 1-μm intervals through the cells in the z-axis.

**Immunogold Electron Microscopy**

Primary oligodendrocytes were grown in filter chambers (Corning) for 3 d before washing them three times with PBS. The cells were fixed in 1% glutaraldehyde and 2% paraformaldehyde for 20 min at room temperature. They were permeabilized and blocked in a similar manner used for confocal immunocytochemistry. OSP/claudin-11 and OAP-1 antibodies were used at 1:50 concentration. The cells were washed and incubated with anti-rabbit IgG conjugated to 12-nm colloidal gold particles (Jackson Immunoresearch Laboratories) for 1 h at room temperature. They were washed and processed by standard methods, and ultrathin cryosectioning was performed as described previously (Bronstein et al., 2000).

**Cell Surface Biotinylation**

Cell surface proteins of primary oligodendrocytes were biotinylated by using a method modified from Mody et al. (1999). In brief, primary oligodendrocyte monolayers were washed and then incubated for 30 min at room temperature in the presence of 1 mM sulfosucinimidyl-6-(biotina-mido)-hexanoate (Supe-h-NHS-Biotin; Pierce Chemical Co.) in PBS. For immunoprecipitation, flasks were washed with PBS and 0.5 M Tris-Cl, and cells were solubilized in buffer A with 1% Triton X-100. Cells were solubilized after washing with PBS and 0.5 M Tris-Cl, centrifuged, and Streapav-din-agarose beads (Sigma-Aldrich) were added to the supernatant. After 3 h of incubation, beads were collected by brief centrifugation and washed. Immunoprecipitated protein was dissociated from the beads by addition of SDS-containing sample buffer with β-mercaptoethanol and DTT and heating for 4 min. The sample was subjected through 12% SDS-PAGE, and electrophoretic protein was probed with anti-OAP-claudin-11 and anti-OAP-1 antibodies.

**Immunoprecipitation and Western Blot Analysis**

Primary oligodendrocytes, CIMO cells, and biotinylated primary oligodendrocytes grown to confluence were washed with 1× PBS. Primary cells and mouse brain homogenates were homogenized in PBS, 0.6 mM PMSF, 1.5 mM EDTA, 0.003% leupeptin, and 0.6 mM DTT. The brain and cell homogenates were subsequently resuspended in buffer A (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100) for 30 min on ice. Insoluble material was pelleted by centrifugation at 18,000 g for 10 min at 4°C. Cen-lular lysate was precleared with 40 μl of protein A (Sephata/agarose) beads for 1 h at 4°C and centrifuged at 3,000 g for 10 min. Specific mole-cules were immunoprecipitated by the incubation of 120-μg protein super-natant with purified primary antibody overnight at 4°C, followed by incu-bation with protein A beads for 1 h at 4°C. The beads were washed three times for 15 min with cold buffer A and resuspended in SDS-PAGE loading buffer. 30 μg of cell homogenate per lane was subjected to SDS-PAGE on 12% (wt/vol) acrylamide gels (Laemmli, 1970). Proteins were transferred to nitrocellulose membranes by semidy electroblotting. Blots were blocked in 5% BSA in TBS-T (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20 [vol/vol]) and then probed with primary antibodies for either 2 h at room temperature or overnight at 4°C. The blots were washed with PBS-T and probed with HRP-conjugated secondary anti-body. Chemiluminescence substrate (Pierce Chemical Co.) was used for the HRP reaction.

**Proliferation Assays**

To evaluate the effect of OSP/claudin-11 and OAP-1 overexpression on proliferation, [3H]thymidine incorporation was measured in CIMO cells stably transfected with either pcDNA3.1 vector alone, pcDNA3.1 containing OAP-1, pBabe vector alone, or pBabe-containing OSP/claudin-11, antisense OSP/claudin-11, PLP, and MBP. Overexpression of OSP/claudin-11, OAP-1, and controls was confirmed by Northern and Western blot analysis. Cells were incubated with [3H]thymidine (0.2 μCi/well) for 16 h under permissive or nonpermissive conditions, washed, and DNA was precipitated with 5% TCA and subsequently determined by a liquid scintilla-tion counter. Incorporated radioactivity was measured in triplicates. All experiments were performed in triplicates and done at least four times.

**Cell Migration Assay**

Cell migration was quantified by measuring the number of cells migrating from the agarose drops using a modification of the method described by Varani et al. (1978). Oligodendrocytes were resuspended at 4 × 10⁵ cells/ml in Sato medium containing 10% heat-inactivated FCS and 0.3% low melting agarose maintained at 37°C to prevent setting of the agarose.
Drops of the cell suspension (2 μl) were applied to the center of wells in a 24-well tissue culture dish, which were then placed at 4°C for 20 min to allow the agarose to solidify. The drops were then covered with 50 μl of Sato medium containing 10 μg/ml fibronectin (Sigma-Aldrich) and incubated for 30 min at 37°C. An additional 350 μl of Sato medium containing platelet-derived growth factor (5 ng/ml) and fibronectin was added to all of the wells with and without antibodies. Cell migration was measured at daily intervals for 1–4 d. At least three experiments were performed and within a single experiment, each condition was tested in four different wells. Statistical significance was assessed by using the Student’s paired t test in which P < 0.05 was considered statistically significant.

Results

A Novel Protein Interacts with the COOH Terminus of OSP/claudin-11

A mouse brain cDNA GAL4 activation domain library was screened with three different OSP/claudin-11 GAL4-binding domain baits in pGBT vector: the entire OSP/claudin-11 ORF, extracellular loop of OSP/claudin-11 between 35 and 84 amino acids, and COOH terminus of OSP/claudin-11 between 122 and 207 amino acids. Only transfection with OSP/claudin-11 COOH terminus bait resulted in positive colonies. Of 2 × 10⁶ transformants screened, 140 colonies grew on His⁺ selection medium; two of these were β-galactosidase positive. None of the negative controls grew on His-deficient medium or activated lacZ transcription. OSP/claudin-11 cotransformed with OAP-1 activated the lacZ reporter in a manner similar to our positive controls of pVA3, pTD1 (murine p53 and SV40 large antigen), and pCL1.

OAP-1 Is a Member of the Tetraspanin Superfamily

OAP-1 cDNA (700 bp) was partially sequenced, and BLAST search revealed that it is a novel clone. The complete OAP-1 cDNA was then isolated from a spinal cord cDNA library and composed of 1,679 bp with an ORF of 762 bp (sequence data available from GenBank/EMBL/DDBJ under accession number AF242591). The predicted protein has 254 amino acid residues and a calculated molecular mass of 28 kD. The original OAP-1 clone isolated from the yeast two-hybrid screen contained nucleotide sequence 778–1053 of the ORF, corresponding to amino acids 162–254 (COOH terminus). Sequence homology and hydropathy plot analysis suggested that OAP-1 belongs to the transmembrane 4 superfamily (TM4SF). TM4SFs include ≥21 different proteins that have four transmembrane-spanning domains and exhibit high homology in 24 conserved regions that are specific for the TM4SF (Todd et al., 1998).
mCD63, mCD82, mTspan-6, and hTspan-3) revealed highly conserved amino acids specific for tetraspanins (Fig. 1 A). There are four sites of potential NH₂-linked glycosylation in extracellular domain 2. For OAP-1 and other TM4SF proteins, conserved sequences are primarily within the putative transmembrane domains analyzed by a Kyte-Doolittle plot (Fig. 1 B). In contrast, the extracellular domains are more divergent in terms of length, sequence, and degree of glycosylation. Sequence similarity between murine OAP-1 and human tetraspanin Tspan-3 suggests that they are homologues.

**OAP-1 mRNA Is Expressed in Oligodendrocytes**

OAP-1 is widely expressed in brain and other tissues. Northern blot analysis revealed a 1.9-kb mRNA present in all the tissues tested, including spinal cord, brain, testes, skeletal muscle, heart, kidney, spleen, and lung (Fig. 2 A). A second band of ~4 kb was also present in much lower levels. This may represent an alternate transcript or another gene product with high homology to OAP-1. Northern blot analysis on RNA from purified primary mouse oligodendrocyte cultures and from the CIMO cell line confirmed the presence of OAP-1 expression in oligodendrocytes (Fig. 2 B and see Fig. 7 A). Western blot analysis revealed a single band of ~31 kD in O2A oligodendrocyte progenitor cells (Fig. 2 C). Two bands of ~31 and 60 kD were present in mature oligodendrocytes and mouse brain homogenates, although under strong reducing conditions (20% β-mercaptoethanol and 50 mM DTT) only the 31-kD band was apparent. The band was eliminated by preincubation of the OAP-1 antibody with peptide (Fig. 2 C). These data suggest that the antibody specifically recognized OAP-1 protein and that the 60-kD band was a dimer of OAP-1 protein.

Using in situ hybridization, we found that OAP-1 mRNA was expressed in all areas of the mouse brain with high levels in hippocampal pyramidal neurons and moderate levels in oligodendrocytes of the corpus callosum (Fig. 3, A and B). Unlike OSP/claudin-11 staining in myelin tracts (Fig. 3 C), immunohistochemistry demonstrated that OAP-1 expression was more widespread, consistent with in situ hybridization experiments (Fig. 3 D).

**OSP/Claudin-11, OAP-1, and β1 Integrin Form a Complex**

To determine if the association of OSP/claudin-11 with OAP-1 occurs in vivo, we performed immunoprecipitation. Homogenates were precipitated under relatively stringent conditions (1% Triton X-100) with either anti-OSP/claudin-11 or anti-OAP-1 antibodies. Western blots of the precipitated proteins confirmed OSP/claudin-11–OAP-1 association in both mouse brain (Fig. 4 A) and primary oligodendrocyte homogenates (Fig. 4 B).

Most if not all TM4SFs (for example, CD81, CD9, CD53, CD63, and CD82) are known to associate with a vast range of integrins in various types of cells (for review see Maecker et al., 1997). This is also true for OAP-1, since it was immunoprecipitated with anti-β1 integrin (Fig. 4, A and B). The
Additional confirmation of an association between OSP/claudin-11, OAP-1, and β1 integrin was obtained using double-labeled immunocytochemistry and confocal microscopy (Fig. 5 A). Controls with GFAP showed no colocalization with any of the experimental proteins (data not shown). A striking colocalization of OSP/claudin-11, OAP-1, and β1 integrin was observed in most oligodendrocytes (Fig. 5 A, third panel in rows 1 and 2). A few oligodendrocytes showed only partial colocalization (data not shown), suggesting that complex formation was under dynamic control or that the population of oligodendrocytes was heterogeneous.

**OSP/claudin-11, OAP-1, and β1 Integrin Is a Surface Membrane Protein Complex**

We predicted that both OSP/claudin-11 and OAP-1 are membrane bound based on their high level of hydrophobicity and known subcellular localization of β1 integrin. Consistent with our hypothesis, OAP-1 and OSP/claudin-11 colocalized on the cell surface of nonpermeabilized live oligodendrocytes immunolabeled before fixation (Fig. 5 B). Surface labeling with anti-OSP antibody was absent in OSP-null oligodendrocytes (data not shown). Furthermore, surface protein biotinylation of nonpermeabilized oligodendrocytes resulted in biotinylation of OSP/claudin-11 and OAP-1 (Fig. 6, A and B). To determine if OSP/claudin-11 and OAP-1 complex on the surface of cells, oligodendrocytes from wild-type (+/+)- and OSP/claudin-11 knockout (−/−) mice were biotinylated and subjected to coimmunoprecipitation. OSP/claudin-11 and OAP-1 protein were detected in OSP/claudin-11 and OAP-1 +/+ immunoprecipitated biotinylated oligodendrocytes (Fig. 6, C and D). Neither OSP/claudin-11 nor OAP-1 protein was detected in −/− oligodendrocytes immunoprecipitated with anti-OSP/claudin-11, although OAP-1 was detected in OAP-1 immunoprecipitated −/− oligodendrocytes (Fig. 6, C and D). Surface localization of these proteins was also confirmed using immunohistochemical EM. Immunoreactive protein localized primarily to the outer cell membranes of primary oligodendrocytes and appeared to be more concentrated on their processes (Fig. 6 E).

**Overexpression of OSP/Claudin-11 and OAP-1 Increases Proliferation in CIMO Cells**

TM4SFs and TJ s have been implicated in the regulation of cellular proliferation. To study the effect of altered OAP-1 and/or OSP/claudin-11 gene expression on growth, CIMO cells were used (Bronstein et al., 1998). Under permissive conditions (33°C + Iry), CIMO cells grew rapidly and were relatively undifferentiated. When transferred to nonpermissive conditions (37°C, lacking Iry), the cells had a more differentiated morphology, grew slowly, and expressed myelin proteins. OSP/claudin-11 mRNA levels were 11-fold higher and OAP-1 levels were 1.5-fold higher in CIMO cells grown under permissive compared with nonpermissive conditions (Fig. 7 A). Increased mRNA expression could have reflected a response to higher cell proliferative rate or may have helped induce proliferation. To distinguish between these possibilities, CIMO cells were transfected with various constructs to alter gene expression, and their effect on proliferation was
measured. CIMO cells overexpressing OSP/claudin-11 protein or OAP-1 protein were confirmed via Western blot analysis (Fig. 7 B). Under permissive conditions, overexpression of OSP/claudin-11 had no significant effect on [3H]thymidine incorporation in CIMO cells nor did overexpression of PLP, MBP, or OSP antisense (in an attempt to lower endogenous OSP/claudin-11) (Fig. 7 C). When the cells were transferred to nonpermissive conditions, the cells overexpressing OSP/claudin-11 incorporated 81-fold more [3H]thymidine compared with those transfected with vector alone and took on a less differentiated appearance. In fact, overexpression of OSP/claudin-11 under these conditions had a 2.5-fold greater proliferative effect than that of a known mitogen, SV40ts58 antigen. There was an increase in cellularity and necessity to passage cells more frequently, consistent with the concept that increase in [3H]thymidine incorporation reflected an increase in proliferation. Thus, OSP/claudin-11 had a powerful proliferative effect on differentiated CIMO cells but not on less differentiated CIMO cells. Interestingly, OAP-1 overexpression of CIMO cells increased proliferation greater than twofold compared with vector alone controls.

Figure 5. OSP/claudin-11 colocalizes with OAP-1 and β1 integrin in oligodendrocytes. (A) Confocal images of primary mouse oligodendrocytes shown in the top row were double labeled for OSP/claudin-11 (Texas red) and OAP-1 (FITC) or β1 integrin (FITC). Fused images are shown at the far right and bottom. Most cells showed striking colocalization of OSP/claudin-11, OAP-1, and β1 integrin. (B) Confocal images of primary mouse oligodendrocytes shown were double labeled for OAP-1 (Texas Red) and OSP/claudin-11 (FITC) in live primary oligodendrocytes. Fused images are showed to the far right. Colocalization of the two proteins was mostly observed in the cell borders. (C) Live primary oligodendrocytes were cultured on slides and immunostained with anti-OAP-1 (top row) and anti-OSP/claudin-11 (bottom row) antibody in the presence and absence of the respective antibody peptides. Background staining is also shown. Bars: (A, top row) 10 μM; (A, bottom row) 25 μM.
under both growth permissive and nonpermissive conditions (Fig. 7 C).

OAP-1–OSP/claudin-11–β1 Integrin Complex Regulates Oligodendrocyte Migration

Cell proliferation, differentiation, and migration are at least partially mediated by the interaction between cell membrane proteins and extracellular matrix (ECM). Integrins are a key family of ECM receptors, and avβ1 integrin has been shown to play a role in oligodendrocyte migration (Milner et al., 1996). Since OSP/claudin-11 and OAP-1 form a complex with β1 integrins, it is likely that they are involved in cellular interactions with ECM. The possible involvement of OSP/claudin-11 and OAP-1 in oligodendrocyte migration was tested using an established in vitro assay used previously to demonstrate that integrins are involved in oligodendrocyte migration (Varani et al., 1978; Milner et al., 1996). Primary oligodendrocytes were plated in an agarose drop in the presence (positive control) or absence (negative control) of fibronectin. Maximal migration in positive controls was achieved in 4 d, with 58 ± 12 cells per well appearing in the migratory zone. Migration of oligodendrocytes was inhibited significantly in the presence of anti–OSP/claudin-11, anti–OAP-1, and anti–β1 integrin antibodies by 95, 76, and 88% of the control, respectively (Fig. 8 A). The inhibitory effect of the antibodies on migration was specific, since inactive anti–OAP-1 (boiled for 10 min) and anti-GFAP (unrelated) antibody had no effect. Reduced migration also did not reflect decreased viability since >95% of all cells under all conditions remained viable as determined by trypan blue staining.

We were surprised by the significant inhibition of migration in the presence of anti–OSP/claudin-11 antibody, since the recognizable epitope is predicted to be intracellular. To confirm that the anti–OSP/claudin-11 antibody was indeed recognizing protein epitopes on live oligodendrocytes, cultures were incubated with anti–OSP/claudin-11 antibodies and with antibody that had been preincubated with OSP/claudin-11 peptide (Fig. 5, B and C). We observed specific labeling of live cells, although not as pronounced as with permeabilized oligodendrocytes (data not shown). This staining was markedly attenuated by preincubation with peptide and was absent in OSP-null oligodendrocytes, demonstrating specificity of the antibody labeling.

Migration assays using oligodendrocytes isolated from OSP/claudin-11-null mice add further support to OSP/claudin-11’s role in migration. Oligodendrocytes completely lacking OSP/claudin-11 had markedly reduced ability to migrate compared with wild-type cells, whereas migration assays using heterozygote cells resulted in intermediate values (Fig. 8 B). Interestingly, only fibronectin-dependent migration was affected by alterations in OSP/claudin-11 gene dosage.

Discussion

Although several proteins have been localized to TJs, none have been shown to associate directly with OSP/claudin-11. Reported here is the identification of an OSP/claudin-11–interacting protein, OAP-1. It is evident from sequence analysis that OAP-1 is a member of the TM4SF. OAP-1, like other members of TM4SF, has (a) four highly conserved hydrophobic transmembrane domains, (b) charged residues in or near the transmembrane domains, and (c) conserved cysteine residues in the second transmembrane domain (Maeker et al., 1997). Tspan-1–Tspan-6 are also new members of the TM4SF family, which were identified by Todd et al. (1998) by searching the dbEST database. OAP-1 shares highest homology with human Tspan-3 and is likely the mouse homologue of human Tspan-3. Like human Tspan-3 mRNA expression, murine OAP-1 showed widespread tissue expression. Within the CNS, OAP-1 localized to oligodendrocytes, neurons, and astrocytes.

Members of TM4SF have been implicated in several basic biological processes (Hemler et al., 1996; Maeker et al.,...
and 37°C (lanes 1 and 3) and 33°C (lanes 2 and 4) probed with a 32P-labeled 2-kb cDNA of OSP/claudin-11 (lanes 1 and 2) and 1.9-kb cDNA of OAP-1 (lanes 3 and 4). Both OSP/claudin-11 and OAP-1 expression is increased at 33°C during proliferation. (B) Western blot of CIMO cells transfected with control vector (lanes 1 and 3) and OAP (lanes 2 and 4) probed with anti-OSP antibody at 33°C (lanes 1 and 2) and 37°C (lanes 3 and 4). (Bottom panel) Western blot of CIMO cells transfected with OAP-1 (lane 1) and control vector (lane 2) at 37°C probed with anti-OAP-1 antibody confirms increased expression in these stable transfecents. (C) [H]Thymidine incorporation into DNA of CIMO cells transfected with control vectors (pBabe and pCDNA) and OSP/claudin-11, antisense OSP/claudin-11, PLP, MBP, and OAP-1 constructs were observed under nonpermissive (37°C, minus I) and permissive conditions (33°C, plus I). OSP/claudin-11–transfected cells had a significant effect on proliferation at 33°C, whereas OAP-1 had a more generalized effect at both 33°C and 37°C. Control CIMO cells incorporated 4,000 ± 800 cpm/well/12 h, similar to the control vector transfected CIMO cells (average ± SEM, n = 24–32; 500 cells/well, each well counted in triplicates). OSP/claudin-11 versus pBabe vector at 37°C (***P < 0.0005), and OAP-1 versus pCDNA vector at 33°C and 37°C (**P < 0.005; Student’s t test).

Figure 7. Overexpression of OSP/claudin-11 and OAP-1 increases proliferation in CIMO cells. (A) Northern blot of CIMO cells grown at 37°C (lanes 1 and 3) and 33°C (lanes 2 and 4) probed with a 32P-labeled 2-kb cDNA of OSP/claudin-11 (lanes 1 and 2) and 1.9-kb cDNA of OAP-1 (lanes 3 and 4). Both OSP/claudin-11 and OAP-1 expression is increased at 33°C during proliferation. (B) Western blot of CIMO cells transfected with control vector (lanes 1 and 3) and OAP (lanes 2 and 4) probed with anti-OSP antibody at 33°C (lanes 1 and 2) and 37°C (lanes 3 and 4). (Bottom panel) Western blot of CIMO cells transfected with OAP-1 (lane 1) and control vector (lane 2) at 37°C probed with anti-OAP-1 antibody confirms increased expression in these stable transfecents. (C) [H]Thymidine incorporation into DNA of CIMO cells transfected with control vectors (pBabe and pCDNA) and OSP/claudin-11, antisense OSP/claudin-11, PLP, MBP, and OAP-1 constructs were observed under nonpermissive (37°C, minus I) and permissive conditions (33°C, plus I). OSP/claudin-11–transfected cells had a significant effect on proliferation at 33°C, whereas OAP-1 had a more generalized effect at both 33°C and 37°C. Control CIMO cells incorporated 4,000 ± 800 cpm/well/12 h, similar to the control vector transfected CIMO cells (average ± SEM, n = 24–32; 500 cells/well, each well counted in triplicates). OSP/claudin-11 versus pBabe vector at 37°C (***P < 0.0005), and OAP-1 versus pCDNA vector at 33°C and 37°C (**P < 0.005; Student’s t test).

Figure 8. Oligodendrocyte migration in the presence of OSP/claudin-11, OAP-1, and β1 integrin antibodies and in OSP/claudin-11–deficient cells. (A) Cells were migrated out of agarose drops in medium: −control, minus fibronectin; + control, plus fibronectin; anti–β1 integrin antibody; anti–OSP/claudin-11 antibody; anti–OAP-1 antibody; boiled inactive anti–OAP-1 antibody. 1 anti–OAP-1; anti–GFAP antibody; GRGDS peptide; and GRGES peptide. Except for the −control, all other conditions contained fibronectin. The total number of cells migrating out of the agar drop was counted after 4 d. Statistical values represent each condition versus positive control: **P < 0.0005; *P < 0.005 (Student’s t test). (B) Migration assays were performed using oligodendrocytes isolated from wild-type (+/+), heterozygous OSP (+/−), and homozygous knockout OSP (−/−) mice, and cells were allowed to migrate in the presence (black bars) and absence (stippled bars) of fibronectin. There was marked attenuation of fibronectin-dependent migration in OSP/claudin-11–deficient cells. Values are expressed as percentage of wild-type. Heterozygous (+/−) versus wild-type (**P < 0.05) and homozygous recessive (−/−) versus wild-type (***P < 0.005; Student’s t test).

1997; Sugiura and Berditchevski, 1999), and it has been proposed that they exert their function by promoting assembly of signaling complexes. TM4SF proteins associate with other TM4SF members, for example, CD4, CD8, PETA-3, NAG-2, and CR2/CD19 (Imai and Yoshie, 1993; Tachibana et al., 1997; Yanez-Mo et al., 1998), and with integrins (Berditchevski et al., 1996; Hadjiargyrou et al., 1996; Macek et al., 1997; Yanez-Mo et al., 1998; Yauch et al., 1998). The discovery that OSP/claudin-11 associated with a member of the TM4SF led us to investigate whether OAP-1 and OSP/claudin-11 formed a complex that includes integrins. Oligodendrocyte precursors in vitro express the integrins α6β1, αvβ1, and αvβ3 subunits (Milner and French-Constant, 1994; Shaw et al., 1996). Because many tetraspansins interact with β1 integrin subunits, we tested the presence and association of β1 integrin subunit with OSP/claudin-11 and OAP-1. The association of these three proteins was first suggested by coimmunoprecipitation in mouse brain homogenates and in primary oligodendrocytes homogenates under relatively stringent conditions. Double immunocytochemistry showed colocalization of OSP/claudin-11, OAP-1, and β1 integrin in the cell body and processes of most oligodendrocytes. Surface biotinylation and immunoelectron microscopy demonstrated the presence of OSP/claudin-11 and OAP-1 on the oligodendrocyte membrane surface, but it is still not clear whether OSP/claudin-11 complexes with OAP-1 and β1 integrin at TJs. This would not be unprecedented, since some members of the TM4SF have been found at cell-to-cell contacts where they are also associated with integrins. For example,
the TM4SFs CD151/PETA-3, CD9, and CD81/TAPA-1 are all localized at intercellular contact sites of endothelial cells (Sincock et al., 1997; Yan-Mo et al., 1998). Experiments involving the localization of OSP/claudin-11, OAP-1, and β1 protein complex in TJ are presently ongoing.

It has been suggested that members of TM4SF and integrins play a role in proliferation, migration, signal transduction, cell activation, and tumor invasion (Hemler et al., 1996; Maeker et al., 1997; Sugiura and Berditchevski, 1999). There is some evidence that at least integrins are involved in some of these processes in oligodendrocytes (Malek-Hedaya and Rome, 1994; Frost et al., 1999; Blaschuk et al., 2000). Data presented here suggest that the OAP-1, OSP/claudin-11, and β1 integrin complex is involved in regulating proliferation and migration of oligodendrocytes. Overexpression of both OSP/claudin-11 and OAP-1 resulted in increased growth in CIMO cells. This finding is consistent with the previously described growth regulatory effects of other TJ-associated proteins (Li and Mrsny, 2000; Ryeom et al., 2000) and the elevated levels of OSP/claudin-11 found in some tumors (Buznikov, A.G., and J.M. Bronstein, unpublished results).

Several lines of evidence support an integral role of integrins and tetraspanins in cellular migration. Integrins have been shown to regulate the migration of a range of neural cells, including neural crest cells (Bronner-Fraser, 1993), Schwann cells (Milner et al., 1997), oligodendrocyte precursors (Milner et al., 1996), and neuronal precursors within the optic tectum (Galileo et al., 1992). The disruption of the β1 subunit gene by homologous recombination has demonstrated the critical role of β1 integrins in migration (Fassler et al., 1995; Hirsch et al., 1996). Antibodies to TM4SF members CD9, CD55, CD81, CD82, CD151/PETA-3, and CD81/TAPA-1 alter integrin-mediated cell migration (Domanico et al., 1997; Lagaudriere-Gesbert et al., 1997; Yan-Mo et al., 1998). Notably, in Schwann cells of the peripheral nervous system, CD9 associates with integrins on the cell surface and an anti-CD9 monoclonal antibody promoted adhesion and proliferation of Schwann cells in vitro (Hadjiajrgou et al., 1996). Given this literature, it was not surprising that antibodies that bind to β1 integrin and OAP-1 proteins caused a profound decrease of oligodendrocyte migration (Fig. 7 A). We did not expect the observed decrease in migration in the presence of anti-OSP/claudin-11 antibody, which was targeted to a putative intracellular COOH termini epitope. To ensure that we were seeing specific binding of this antibody, we performed peptide-blocking experiments proving that the antibody was recognizing this sequence. It is possible that it is also recognizing another epitope, but this is highly unlikely since immunohistochemical staining using this antibody is specific and almost identical with that described by Morita et al. (1999) and staining is absent in OSP-null cells. The specificity of the inhibitory response is supported by the fact that an unrelated antibody or inactivated antibody had no effect on migration. Thus, the predicted structure of OSP/claudin-11 is wrong or is dynamic or the antibody is gaining access to an intracellular epitope. Given the potential importance of our finding that anti-OSP/claudin-11 antibodies inhibit migration and the unexpected staining of live cells, we felt it was essential to confirm OSP/claudin-11’s role in migration using another technique. The alterations in migratory behavior of oligodendrocytes in the OSP-null mouse and the dose dependency of this effect add strong support for our conclusion. It is possible that these results reflected altered proliferation rates, but we feel this is unlikely. Oligodendrocyte cell cultures from transgenic animals appeared similar to wild-type cultures, and myelin appears normal in the adult OSP/claudin-11 knockout mouse (Gow et al., 1999). Although quantitation of the number of oligodendrocytes has not been performed in these mice, it is possible that OSP/OAP-1/integrin-mediated migration is not required for relatively normal myelination to occur during development. Experiments are underway evaluating OSP/claudin-11’s role in proliferation and migration during remyelination after injury.

The formation of TJ is normally associated with a cellular quiescent nonproliferating and nonmigratory state. Conversely, integrin/TM4SF activation has been positively associated with cell growth and migration, suggesting that the interaction of OSP/claudin-11 with this complex may therefore be a dynamic one. These associations could also be important in cytoskeletal interactions and interactions with different regulatory proteins. Further studies are necessary to determine the importance of OSP/claudin-11/OAP-1/integrin association in oligodendroglial proliferation and migration during myelin formation, maintenance, and repair and to determine if this mechanism can be generalized to other members of the claudin family.

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