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.,

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[1]*Abbreviations used in this paper:* CNS, central nervous system; ECM, extracellular matrix; MBP, myelin basic protein; OAP, OSP-associated protein; ONPG, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; OSP; oligodendrocyte-specific protein; PLP, proteolipid protein; SD, synthetic dropout; TJ, tight junction; TM4SF, transmembrane 4 superfamily.

1999). OSP/claudin-11 appears to be necessary for the formation of TJs as OSP/claudin-11–null mice lack intramembranous junctions in between Sertoli cells in testes and within CNS myelin sheaths, leading to reproductive and neurological deficiencies (Gow et al., 1999). Indeed, OSP/claudin-11 knockout mice have slowed CNS conduction velocities, suggesting that OSP/claudin-11–containing TJs are necessary for normal axonal function (Gow et al., 1999). It is not yet known if the expression of OSP/claudin-11 alone is sufficient to establish the TJ molecular barrier or regulate intramembrane diffusion of lipids.

Three types of integral membrane proteins that localize to TJs in vivo have been described: occludin (Furuse et al., 1993), claudins (Furuse et al., 1998), and junction adhesion membrane protein (Martin-Padura et al., 1998). It is likely that these proteins are involved in establishing the barrier function of TJs, although OSP/claudin-11 is the only protein to date to be found essential for the formation of some intramembrane strands (Gow et al., 1999). Several other proteins have been identified which localize to the cytoplasmic membrane region of TJs: ZO-1 (Stevenson et al., 1986), ZO-2 (Jesaitis and Goodenough, 1994), ZO-3 (Haskins et al., 1998), cingulin (Citi et al., 1988), 7H6 (Zhong et al., 1993), symplekin (Keon et al., 1996), AF-6/afadin (Yamamoto et al., 1997), and some Rab proteins (Weber et al., 1994; Zahraoui et al., 1994). These proteins may mediate other proposed functions of TJ proteins such as regulating cell growth, transformation, and differentiation (Willott et al., 1993; Li and Mrsny, 2000; Reichert et al., 2000; Ryeom et al., 2000). It has been proposed that the cytoplasmic proteins associate with one or more of the integral TJ membrane proteins, although direct evidence is lacking. ZO-1 can associate with occludin in vitro (Furuse et al., 1994), although it continues to localize to TJs in the occludin-deficient stem cells (Saitou et al., 1998) and in the testes of the OSP/claudin-11 gene knockout mice (Gow et al., 1999). A recent report has demonstrated direct binding of ZO-1, ZO-2, and ZO-3 with the COOH termini of claudins 1–8 in vitro (Itoh et al., 1999). This interaction appears to be dependent on the PDZ domains of ZO-1, 2, and, 3 and a COOH-terminal YV amino acid sequence of many of the claudins (Itoh et al., 1999). Unlike other claudins, OSP/claudin-11 terminates with HV, and no proteins have been identified to associate with it.

Since known TJ proteins apparently do not associate with OSP/claudin-11, we have used the yeast two-hybrid system to identify associated proteins. A novel member of the tetraspanin super family (TM4SF) was isolated, which interacted with the COOH termini of OSP/claudin-11. The putative interaction was examined by coimmunoprecipitation and by immunohistochemistry colocalization. We have named this protein OSP/claudin-11–associated protein 1 (OAP-1). OAP-1 forms a complex with OSP/claudin-11 and β1 integrin, and evidence is provided to support the role for this complex in regulating proliferation and migration of oligodendrocytes.

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 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.

Yeast Two-Hybrid Screening

100 μg of library DNA and OSP/claudin-11 bait were cotransformed into HF7c-competent yeast cells. Transformation of *Saccharomyces cerevisiae* was performed using the lithium acetate method (Gietz et al., 1992). Two reporter genes for interacting proteins are contained in the Hfc7 strain: HIS3 and β-galactosidase. Transformants which expressed interacting proteins grew on histidine-dependent medium (His^-); all positive clones were transferred to sterile Whatman no. 5 filters, placed on selection Trp^-/ Leu^-/His^- minimal synthetic dropout (SD) medium agar, incubated for 1–3 d at 30°C, and fixed by freezing in liquid nitrogen. Filters were then placed onto filter paper presoaked in buffer Z (100 mM NaH_2PO_4, pH 7.0, 10 mM KCl, 1 mM $MgSO_4$), supplemented with 50 mM β-mercaptoethanol and 0.07 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (ONPG) (Sigma-Aldrich). Filters were then incubated at 30°C and monitored for the appearance of blue colonies.

Plasmids from positive yeast colonies were isolated by Yeastmaker (a yeast plasmid isolation kit) (CLONTECH Laboratories, Inc.) method. In brief, yeast cells were spread onto His^-/Leu^-/Trp^- SD plates and incubated at 30°C for 3–4 d. 10-mm^2 portions of the yeast patch were scraped and resuspended in Lyticase containing Tris-EDTA solution at 37°C for 60 min to destroy yeast cell walls, followed by the addition of 20% SDS, vortexed, and loaded onto Chroma spin-1000 columns. Columns were centrifuged at 700 *g*, and elutes containing plasmids were collected and stored at −20°C. Plasmids isolated from positive colonies were individually cotransformed with OSP/claudin-11 bait cDNA and plated on His^- SD medium and tested for β-galactosidase activity as described above. Further confirmation of positive clones was also performed in SFY526 cells using a liquid β-galactosidase assay, using ONPG as substrate. For this assay, SFY526 yeast cells were transformed with candidate and bait plasmids, grown overnight in Leu^-/Trp^- SD medium mixed with YPD medium (CLONTECH Laboratories, Inc.), centrifuged, resuspended in buffer Z, and frozen in liquid nitrogen, followed by an incubation with 0.07% ONPG for 2–5 h. Optical densities were measured at wavelengths of 420 and 600 nm.

Several controls were used to elucidate false positives. Positive controls included cotransformation of pVA3-murine $p53_{(72-390)}$ in pGBT9, with pTD1-SV40 large T-$antigen_{(84-708)}$ in pGAD3F and transformation of pCL1 (wild-type full-length GAL4) (Fields and Song, 1989; Chien et al., 1991). Negative controls included cotransformation of candidate clones with pGAD GH library cloning vector, murine MBP in pGAD GH, and pTD1 clone. Cotransformants were assayed for their ability to grow on His^- medium and for β-galactosidase activity.

Northern Blot Analysis

Total RNA was prepared from different mouse tissues as described by Chomczynski and Sacchi (1987). Total RNA (10 μg/lane) was separated electrophoretically on 1% agarose-formaldehyde gels, transferred to Hybond™-N^+ nylon membranes, and irreversibly fixed by incubating filters at 80°C for 2 h. Prehybridization and hybridization were performed at 65°C in solutions containing 7.5% SDS, 0.5 M phosphate buffer, pH 7.0, 1 mM EDTA, and 1% bovine serum albumin. ^{32}P-labeled ORF OAP-1 cDNA probes were generated by isolating cDNA from plasmid DNA and using random primers according to the manufacturer's protocol (New England Biolabs, Inc.). After hybridization, membranes were washed with 0.2× SSC and 0.1% SDS at 61°C before exposure to x-ray film. Northern blots were normalized with radiolabeled 18S probe (Ambion).

In Situ Hybridization

In situ hybridization was performed on adult mouse brains as described previously (Kornblum et al., 1994). In brief, an adult mouse was perfused fixed before sectioning of the brain. The probes used for localization studies were obtained by in vitro transcription in the presence of [^{35}S]UTP from linearized pGEM 7Zf plasmids containing mouse OAP-1 cDNA. Hybridization was carried out at 60°C overnight in a mixture containing 50% formaldehyde, 10% dextran sulfate, 0.7% ficoll, 0.7% polyvinyl pyrrolidone, 0.7% BSA, 0.15 mg/ml yeast transfer RNA, 0.33 mg/ml denatured salmon sperm DNA, 40-mM DTT, and ^{35}S-labeled cRNA at a con-

centration of 10^7 cpm/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 thiosulfate. 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 for 2–8 wk. 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 (Iγ; 100 U/ml) (GIBCO BRL) at 33°C or 37°C in 5% CO_2. Cells were either maintained in medium with Iγ at 33°C (permissive) or 37°C in medium lacking Iγ (nonpermissive). CIMO cells were stable-transfected with pBabe vector, OSP/claudin-11, antisense 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 genetecin 500 μg/ml for pcDNA vector background) selection.

Purification of Oligodendrocytes. Purified oligodendrocytes were obtained using 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-YSDWENTDWFKETKNQSV-OH) conjugated to keyhole limpet 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 horseradish-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 (1:3,000) and FITC (1:2,000) 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 coverslipped 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 sulfosuccinimydyl-6 (biotinamido)-hexanoate (Sulpho-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 Strepavidin-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 electroblotted protein was probed with anti–OSP/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. Cellular lysate was precleared with 40 μl of protein A (Sepharose/agarose) beads for 1 h at 4°C and centrifuged at 3,000 *g* for 10 min. Specific molecules were immunoprecipitated by the incubation of 120-μg protein supernatant with purified primary antibody overnight at 4°C, followed by incubation 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 semidry 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 TBS-T and probed with HRP-conjugated secondary antibody. 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, [^{3}H]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 [^{3}H]thymidine (0.2 mCi/well) for 16 h under permissive or nonpermissive conditions, washed, and DNA was precipitated with 5% TCA and subsequently determined by a liquid scintillation 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 4×10^7 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.

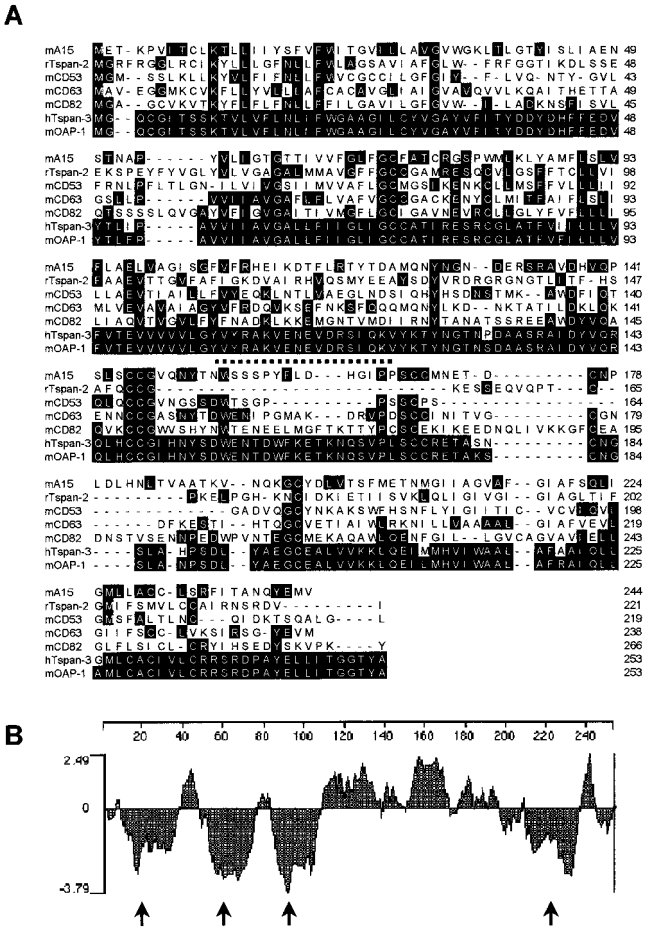

Figure 1. OAP-1 belongs to the tetraspanin family of proteins. (A) Multiple sequence alignment of OAP-1 with murine tetraspanins (mA15, mCD53, mCD63, mCD82), a rat tetraspanin (rTspan-2), and a human tetraspanin (hTspan-3). Boxed residues denote conservation among TM4SFs. (B) Kyte-Doolittle hydropathy plot of the predicted amino acid sequence of OAP-1, predicting four-transmembrane hydrophobic domains (arrows).

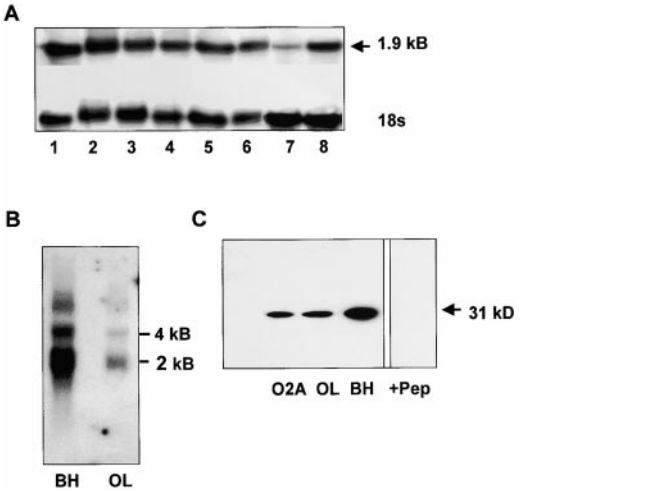

Figure 2. Presence of OAP-1 mRNA and protein in mouse brain and oligodendrocytes. (A) Northern blot analysis of OAP-1 expression. mRNA isolated from normal spinal cord (lane 1), brain (2), testes (3), skeletal muscle (4), heart (5), kidney (6), spleen (7), and lung (8) was probed with a ^{32}P-labeled 1.9-kB cDNA of OAP-1. OAP-1 RNA was abundant in all tissue types. Gel loading was quantified with 18S expression. (B) Northern blot analysis of OAP-1 expression in mouse brain homogenate (BH) and purified oligodendrocytes (OL) from mouse brain. (C) Western blot analysis of OAP-1 protein in oligodendrocyte progenitor cells (O2A), purified mouse oligodendrocytes, and mouse brain homogenate. Preincubation of antibody with OAP-1–specific peptide eliminated the 31-kD band confirming antibody specificity (+Pep).

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 2×10^6 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 termini). 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). Alignment of the OAP-1 sequence with some murine and human TM4SF members (mA15, mCD53,

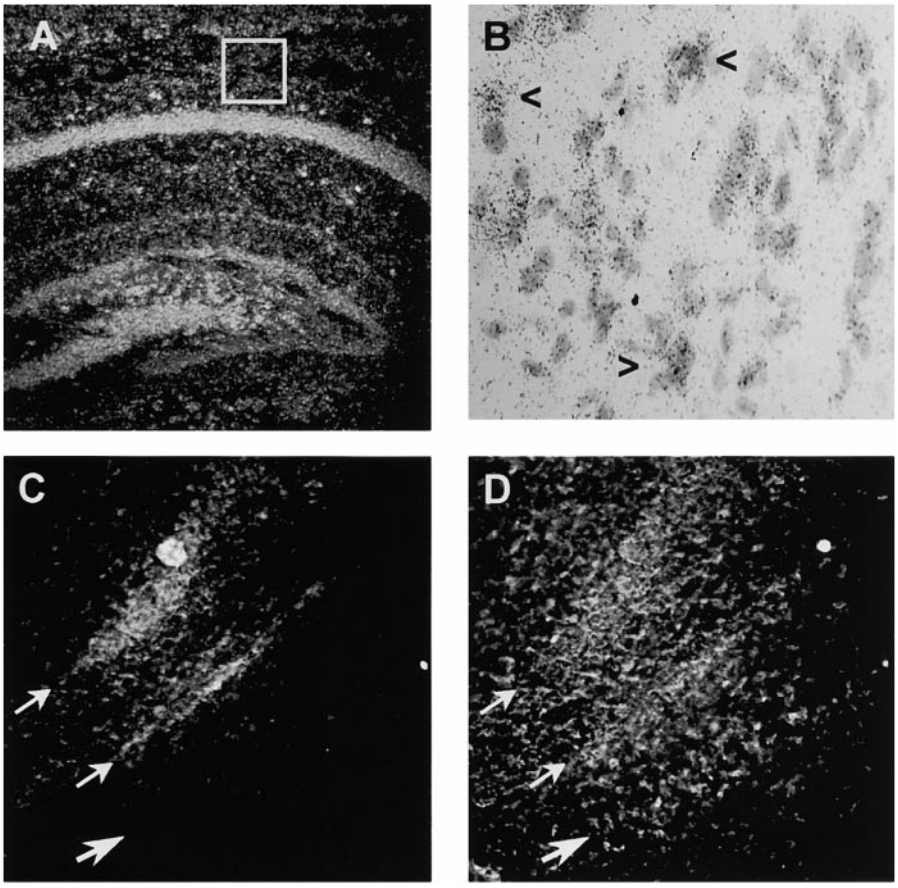

Figure 3. In situ hybridization and immunohistochemistry showing OAP-1 expression in mouse forebrain. (A) Dark field photomicrograph of adult rat forebrain after in situ hybridization using ^{35}S–OAP-1 cRNA shows dense neuronal expression of OAP-1 mRNAs in the CA1 region of a P10 mouse hippocampus. The area under the square is shown in B. (B) A high power bright field photomicrograph showing expression of OAP-1 mRNA in oligodendrocytes of the corpus callosum (arrowheads). (C) Using an immunohistochemistry anti–OSP/claudin-11 antibody (Texas red secondary antibody) showed staining primarily in white matter tracts and not in pyramidal cells and astrocytes (large arrow). Small arrows denote the outer borders of corpus callosum. (D) In contrast to OSP/claudin-11, immunohistochemistry using anti–OAP-1 antibody (FITC secondary antibody) in the same section demonstrated widespread staining in white matter tracts and in areas containing pyramidal cells and astrocytes (large arrow).

mCD63, mCD82, mTspan-6, and hTspan-3) revealed highly conserved amino acids specific for tetraspanins (Fig. 1 A). There are four sites of potential NH_2-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

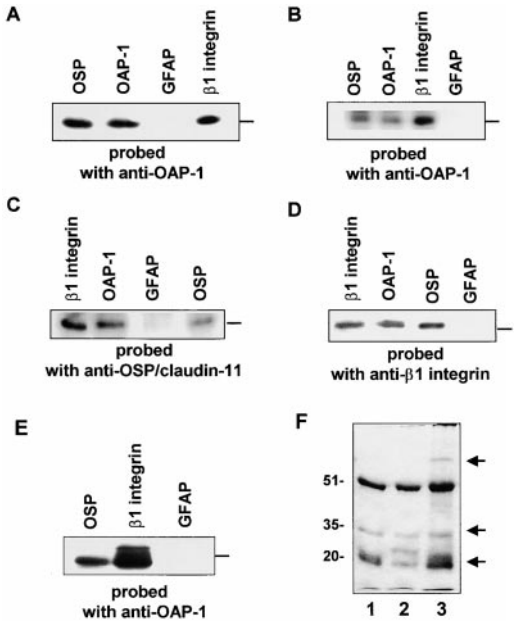

Figure 4. OAP-1 associates with OSP/claudin-11 and β1 integrin. (A) Mouse brain homogenates were immunoprecipitated with anti-OSP, anti–OAP-1, anti-GFAP, or anti–β1 integrin and then probed with anti–OAP-1 antibody. (B) Primary oligodendrocyte homogenates were immunoprecipitated with anti-OSP, anti–OAP-1, anti-GFAP, or anti–β1 integrin and then probed with anti–OAP-1 antibody. (C) Mouse brain homogenates were immunoprecipitated with anti-OSP, anti–OAP-1, anti-GFAP, or anti–β1 integrin and then probed with anti–OSP/claudin-11 antibody. (D) Mouse brain homogenates were immunoprecipitated with anti-OSP, anti-OAP-1, anti-GFAP, or anti–β1 integrin and then probed with anti–β1 integrin antibody. (E) CIMO homogenates were immunoprecipitated with anti-OSP, anti–β1 integrin, or anti-GFAP and then probed with anti–OAP-1 antibody. (F) Silver-stained gel of anti–OSP/claudin-11 (lane 1), anti–OAP-1 (lane 2), and anti–β1 integrin (lane 3) immunoprecipitated mouse brain homogenate. Small arrows denote bands that correspond to the molecular masses of OAP-1, OSP/claudin-11, and β1 integrin. The IgG band is visible at ~50 kD.

complementary experiments indicated that OSP/claudin-11 (Fig. 4 C) and β1 integrin (Fig. 4 D) can be immunoprecipitated from mouse brain homogenates with anti–OAP-1. Furthermore, OSP/claudin-11 and OAP-1 were immunoprecipitated with anti–β1 integrin antibody, and β1 integrin was immunoprecipitated with anti–OSP/claudin-11 antibody (Fig. 4). The identity of β1 integrin on the Western blots was confirmed using both polyclonal and monoclonal antibodies. OSP/claudin-11, OAP-1, and β1 integrin also coimmunoprecipitated in CIMO cell line under permissive and nonpermissive conditions (Fig. 4 E). Neither β1 integrin, OAP-1, nor OSP/claudin-11 was precipitated when the primary antibody was omitted or when an unrelated antibody GFAP was used as the primary antibody. Silver staining of OSP/claudin-11, OAP-1, and β1 integrin immunoprecipitated mouse brain homogenates showed two to four bands corresponding to the molecular masses of OSP/claudin-11, OAP-1, and β1 integrin along with the IgG band at ~50 kD (Fig. 4 F). These results suggest that OAP-1, OSP/claudin-11, and β1 integrin form a complex in the brain and oligodendrocyte homogenates.

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 or 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 TJs 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 + Iγ), CIMO cells grew rapidly and were relatively undifferentiated. When transferred to nonpermissive conditions (37°C, lacking Iγ), 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 (Fig. 7 A) 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

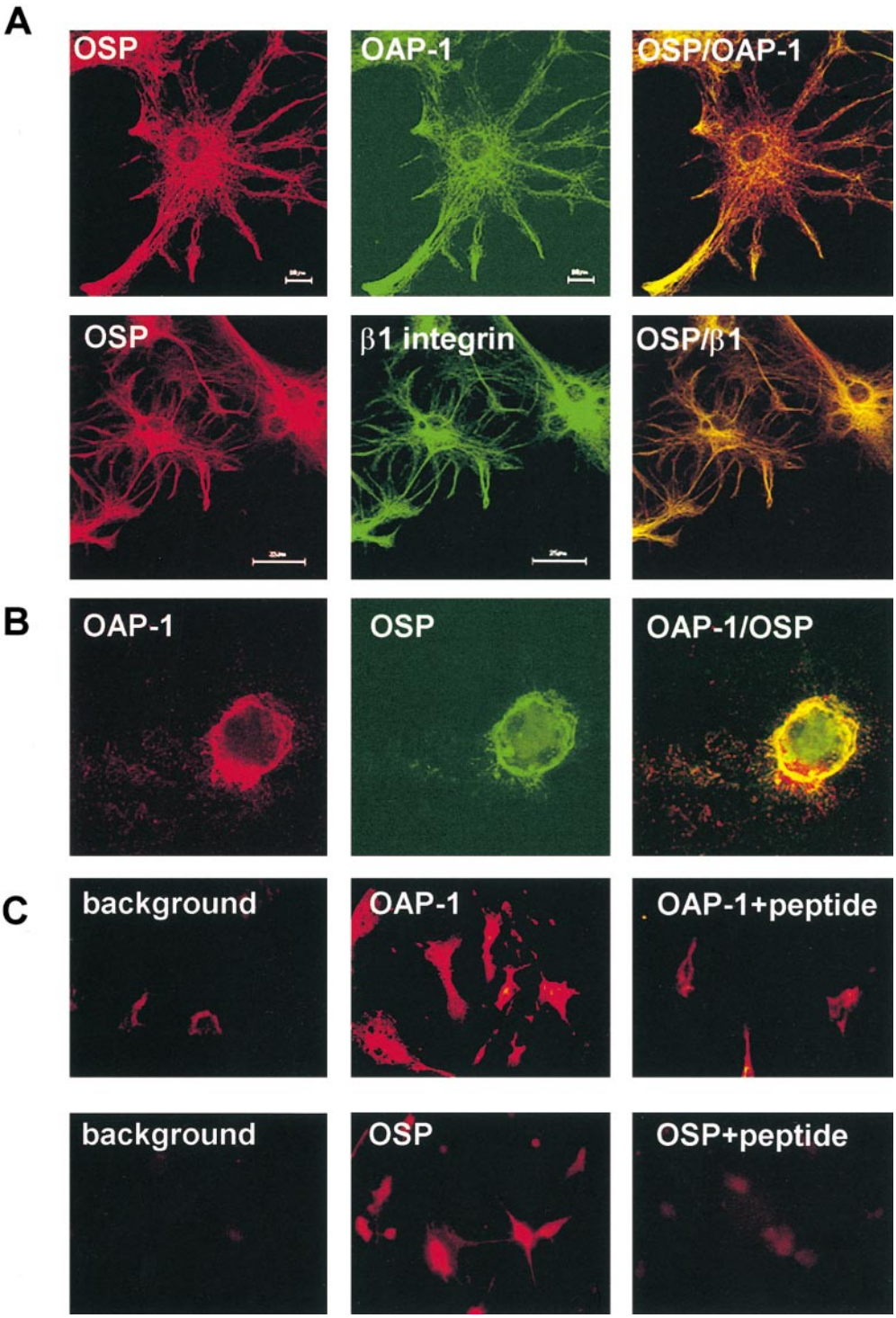

Figure 5. OSP/claudin-11 colocalizes with OAP-1 and β1 integrin in oligodendrocytes. (A) Confocal images of primary mouse oligodendrocytes shown in the top row were double labeled for OSP/claudin-11 (Texas red) and OAP-1 (FITC) or β1 integrin (FITC). Fused images are shown at the far right and bottom. 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.

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 [^{3}H]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 [^{3}H]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 [^{3}H]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

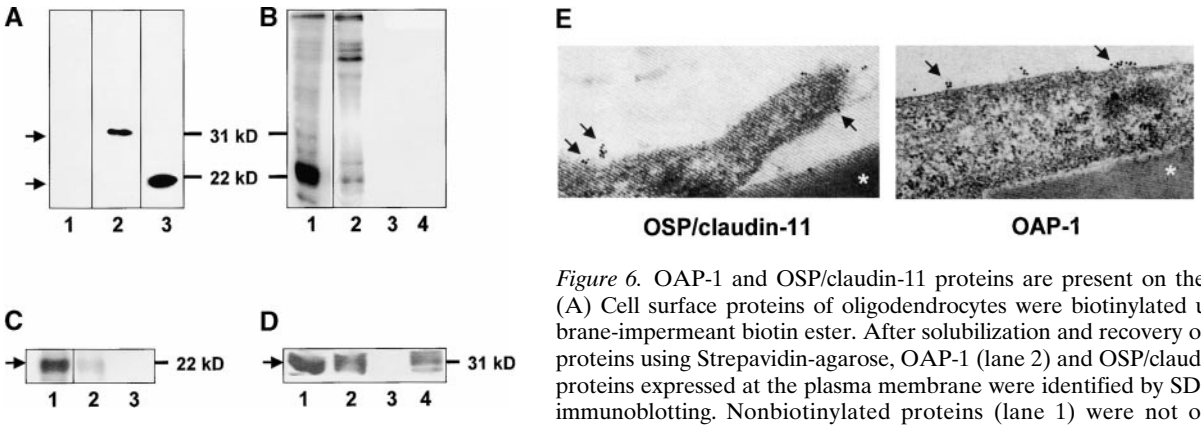

Figure 6. OAP-1 and OSP/claudin-11 proteins are present on the cell surface. (A) Cell surface proteins of oligodendrocytes were biotinylated using a membrane-impermeant biotin ester. After solubilization and recovery of biotinylated proteins using Strepavidin-agarose, OAP-1 (lane 2) and OSP/claudin-11 (lane 3) proteins expressed at the plasma membrane were identified by SDS-PAGE and immunoblotting. Nonbiotinylated proteins (lane 1) were not observed. (B) Surface biotinylated oligodendrocyte proteins isolated from wild-type and OSP/claudin-11 transgenic mice were immunoprecipitated with anti–OAP-1 antibody (+/+, lane 1) and anti–OSP/claudin-11 antibody (+/+, lane 2; −/−, lane 3). Nonbiotinylated protein from wild-type oligodendrocytes was precipitated with anti–OSP/claudin-11 antibody (lane 4). Biotinylated proteins were visualized using Streptavidin-HRP as described in Materials and Methods. (C) Surface-biotinylated primary oligodendrocyte homogenates from wild-type and OSP/claudin-11 transgenic mice were immunoprecipitated with anti–OAP-1 antibody (+/+, lane 1) or anti–OSP/claudin-11 antibody (+/+, lane 2; −/−, lane 3) and probed with anti–OSP/claudin-11 antibody. (D) Surface-biotinylated primary oligodendrocyte homogenates from wild-type and OSP/claudin-11 transgenic mice were immunoprecipitated with anti–OAP-1 antibody (+/+, lane 1) or anti–OSP/claudin-11 antibody (+/+, lane 2; −/−, lane 3) and probed with anti–OAP-1 antibody. (E) Immunohistochemical EM of primary oligodendrocytes. OSP/claudin-11 and OAP-1 immunoreactivity was localized to the outer cell membrane (arrows). The asterisks represent the filter matrix the oligodendrocytes were grown on.

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 αvβ1 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 (Maecker 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; Maecker et al.,

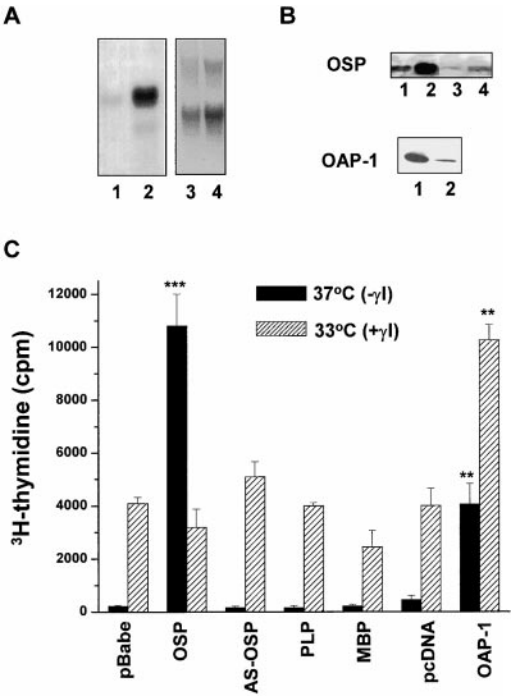

Figure 7. Overexpression of OSP/claudin-11 and OAP-1 increases proliferation in CIMO cells. (A) Northern blot of CIMO cells grown at 37°C (lanes 1 and 3) and 33°C (lanes 2 and 4) probed with a ^{32}P-labeled 2-kb cDNA of OSP/claudin-11 (lanes 1 and 2) and 1.9-kB cDNA of OAP-1 (lanes 3 and 4). Both OSP/claudin-11 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 OSP (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 transfectents. (C) [^{3}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 Iγ) and permissive conditions (33°C, plus Iγ). 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).

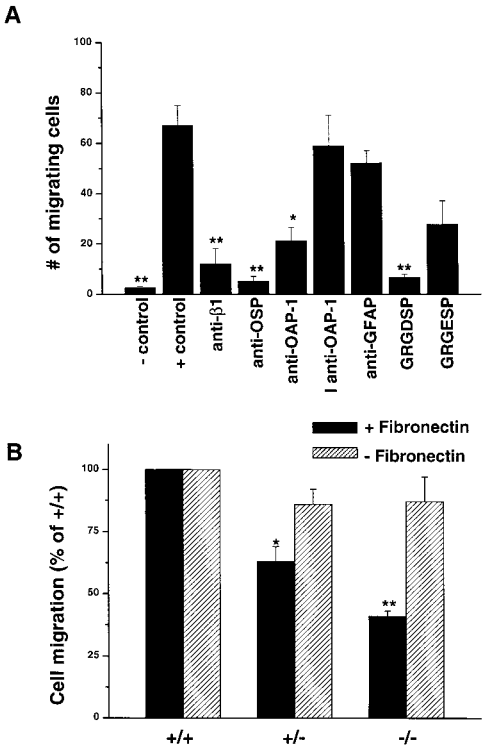

Figure 8. Oligodendrocyte migration in the presence of OSP/claudin-11, OAP-1, and β1 integrin antibodies and in OSP/claudin-11–deficient cells. (A) Cells were migrated out of agarose drops in medium: −control, minus fibronectin; +control, plus fibronectin; anti–β1 integrin antibody; anti–OSP/claudin-11 antibody; anti–OAP-1 antibody; boiled inactive anti–OAP-1 antibody, I anti–OAP-1; anti-GFAP antibody; GRGDSP peptide; and GRGESP 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; Maecker 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 Ffrench-Constant, 1994; Shaw et al., 1996). Because many tetraspanins 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; Yanez-Mo et al., 1998). Experiments involving the localization of OSP/claudin-11, OAP-1, and β1 protein complex in TJs 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; Maecker 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-Hedayat 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 precursor (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, CD53, CD81, CD82, CD151/PETA-3, and CD81/TAPA-1 alter integrin-mediated cell migration (Domanico et al., 1997; Lagaudriere-Gesbert et al., 1997; Yanez-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 (Hadjiargyrou 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 TJs 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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