Purification and Analysis of in Vivo-Differentiated Oligodendrocytes Expressing the Green Fluorescent Protein

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A complete understanding of the molecular mechanisms involved in the formation and repair of the central nervous system myelin sheath requires an unambiguous identification and isolation of in vivo-differentiated myelin-forming cells. In order to develop a novel tool for the analysis of in vivo-differentiated oligodendrocytes, we generated transgenic mice expressing a red-shifted variant of the green fluorescent protein under the control of the proteolipid protein promoter. We demonstrate here that green fluorescent protein-derived fluorescence in the central nervous system of 9-day- to 7-week-old mice is restricted to mature oligodendrocytes, as determined by its spatiotemporal appearance and by both immunocytochemical and electrophysiological criteria. Green fluorescent protein-positive oligodendrocytes could easily be visualized in live and fixed tissue. Furthermore, we show that this convenient and reliable identification now allows detailed physiological analyses of differentiated oligodendrocytes in situ. In addition, we developed a novel tissue culture system for in vivo-differentiated oligodendrocytes. Initial data using this system indicate that, for oligodendrocytes isolated after differentiation in vivo, as yet unidentified factors secreted by astrocytes are necessary for survival and/or reappearance of a mature phenotype in culture.

Key Words: GFP; differentiated oligodendrocyte; patch clamp; primary culture; live cell imaging.

INTRODUCTION

A better understanding of the molecular mechanisms by which the oligodendrocyte forms and maintains the central nervous system (CNS) myelin sheath likely advances the development of strategies for repair of the injured sheath in demyelinating diseases, such as multiple sclerosis (Duncan et al., 1997; Franklin and Blakemore, 1997; Lassmann et al., 1997). Oligodendrocyte differentiation has been investigated extensively in cell culture systems. While these studies have provided excellent insights into the biology of the early stages of the oligodendrocyte lineage (Noble and Wolswijk, 1992; Barres and Raff, 1994), investigations of oligodendrocytes differentiated in culture might reveal functional characteristics that are different from those existing in situ in the brain, and there are likely differences between the initial differentiation of the oligodendrocyte and remyelination in the adult.

In order to provide unequivocal identification and purification of in vivo-differentiated oligodendrocytes, we generated transgenic mice expressing a red-shifted variant of the green fluorescent protein (GFP) under the control of the proteolipid protein promoter.
proteolipid protein (PLP) promoter (PLP-GFP mice). PLP is a gene that in the CNS is specifically expressed in differentiating and mature oligodendrocytes. Using a promoter construct that contains all the information required for spatio-temporal expression similar to PLP, we previously generated transgenic mice that express a PLP–β-galactosidase fusion protein (Wight et al., 1993). Although these mice provide a valuable source of tagged oligodendrocytes, the requirement of an enzymatic reaction or highly specific antibodies precludes their use for visualization in live tissue or purification of oligodendrocytes from cell suspensions. In contrast, GFP yields a bright green fluorescence in the absence of cofactors and in a species-suspensions. In contrast, GFP yields a bright green fluorescence.

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In the current study, transgenic mice (PLP-GFP mice) in which mature oligodendrocytes are specifically labeled by GFP-derived fluorescence have been generated. Electrophysiological analyses in these mice show that this cell-type-specific fluorescent label provides an excellent tool for detailed physiological characterization of differentiated oligodendrocytes in situ. Furthermore, these mice are a novel source for the isolation of in vivo-differentiated oligodendrocytes and, therefore, for a unique tissue culture system that allows the characterization of potentially novel factors necessary for survival and/or maturation of in vivo terminally differentiated oligodendrocytes. Ultimately, the use of the transgenic PLP-GFP mice permits a variety of new approaches for the analysis of functional characteristics and regenerative capacities of mature oligodendrocytes.

**MATERIALS AND METHODS**

**Generation of Transgenic Mice**

For the generation of the transgenic PLP-GFP construct, the PLP-LaCZ construct used by Wight et al. (1993) was modified as follows: The ATG start codon for the PLP protein in exon 1 was mutated using the Sculptor in vitro mutagenesis system (Amer- sham, Arlington Heights, IL), which is based on the phosphorothioate technique (Sayers et al., 1992; Taylor et al., 1985). The sequence around the ATG was changed from GAC ATG to CTC GAG (CAGA GTG CCA AAC CTC GAG GTG AGT TTC). This mutation created an additional XhoI site for better analysis and allowed protein translation to start at the appropriate ATG in the GFP mRNA, while keeping PLP exon/intron consensus sequences that are known to be necessary for proper splicing. An additional ATG located at the beginning of exon 2 was mutated using the USE mutagenesis kit (Pharmacia, Piscataway, NJ) which is based on the unique-site elimination mutagenesis procedure (Deng and Nick- oloff, 1992). The target mutagenic primer oligonucleotide (G TTA GAG TGT GCC GCA TGC TGT CTG GTA G) introduced an SphI site, which was used to delete the sequences coding for the first 13 amino acids of PLP and to insert polylinker sequences that included the unique restriction sites SfiI, Smal, EcoRV, NotI, and SacI. Subsequently, the SV40 poly(A) sequence derived from pCDNA3 (Invitrogen, Carlsbad, CA) (nt 2933–3128) was introduced as a Smal–NotI fragment (amplified by PCR to introduce 5' and 3' restriction sites). Further unique restriction sites (such as Ascl and PacI) were introduced by insertion of the Smal–HindIII fragment derived from the polylinker region of the vector pN6B193 (New England Biolabs, Inc., Beverly, MA). The sequence coding for GFP was isolated from the vector phGFPS65T (Clontech Laboratories, Inc., Palo Alto, CA) as a 734-bp HindIII–NotI fragment. To enable insertion of the GFP sequences into the restriction sites Ascl and PacI, a modified version of the vector pN6B193 was generated. Briefly, after deletion of the sequences located between EcoRI and Smal, as well as XbaI and HindIII, the following restriction sites were inserted between the Ascl and the PacI sites: HindIII, PstI, Smal, NotI, and Xhol. After subcloning into this intermediate vector, the GFP sequences could be inserted as a 770-bp Ascl–PacI fragment.

For injection into pronuclei of B6CBA fertilized oocytes, an approximately 12-kb Apal–SacI fragment (see Fig. 1) was used. Oocytes were transferred into oviducts of pseudopregnant mice (Hogan et al., 1994), and the resulting potential founder animals were tested by Southern blot analysis (Sanbrook et al., 1989). Genomic DNA was prepared from tail biopsies using the QIAamp tissue kit (Qiagen Inc., Valencia, CA). One male founder was identified and bred to C57/B6 females to generate a homozygous line.

**Northern Blot Analysis**

RNA was isolated from different tissues of wild-type and transgenic PLP-GFP mice using the single-step RNA isolation method described by Chomczynski and Sacchi (1987). Ten micrograms of total RNA were separated on 1.2% agarose gels containing 2.2 M formaldehyde. RNA was transferred to MagnaGraph nylon membranes (Micron Separations, Inc., Westboro, MA) and hybridized sequentially with the complete cDNAs coding for GFP (phGFPS65T; Clontech Laboratories, Inc., PLP (LeVine et al., 1990; Sorg et al., 1987), and cyclophilin (Danielson et al., 1988) at 42°C. DNA fragments were labeled with [α-32P]dCTP using the High Prime labeling kit according to the manufacturer’s instructions (Boehringer Mannheim, Indianapolis, IN). Analysis was performed by autoradiography and phosphorimaging techniques (Molecular Dynamics, Sunnyvale, CA). After each analysis, blots were stripped using two 30-min incubations in 5 mM Tris–HCl, pH 7.5, 0.2 mM EDTA, pH 8.0, 0.1× Denhard’ts, 0.05% pyrophosphate at 65°C and then reused for further hybridizations.

**Western Blot Analysis**

Tissues from 23-day-old transgenic PLP-GFP mice were homogenized in 50 mM Tris–HCl, pH 7.5, 2 mM EGTA in the presence of protease inhibitors (1 μg/ml leupeptin (Sigma, St. Louis, MO), 1 mg/ml Pefabloc (Boehringer Mannheim)) using a Polytron homogenizer (Brinkmann Instruments, Inc, Westbury, NY). Protein concentrations were determined by the bicinchoninic acid protein assay (Sigma). Homogenates were diluted 1:2 in sample buffer (2% SDS, 20% glycerol, 100 mM DTT, 50 mM Tris, pH 6.8, 0.1% bromphenol blue) and proteins were denatured by boiling. Forty micrograms of each protein sample was
resolved on 4–20% SDS/polyacrylamide gels (Novex, San Diego, CA). Purified recombinant GFP-S65T protein (Clontech Laboratories, Inc.) was used as positive control. Proteins were transferred to Immobilon-P PVDF membranes (Millipore Corp., Bedford, MA) in Tris/glycine/methanol buffer at 350 mA for 2 h (Towbin et al., 1979). Membranes were blocked in 5% fat-free milk powder in TBS-T (10 mM Tris–HCl, pH 7.5, 0.15 M NaCl, 0.2% Tween 20) and incubated overnight at 4°C with polyclonal anti-GFP antibodies (Clontech Laboratories, Inc.) or polyclonal anti-actin antibodies (Sigma) at a dilution of 1:1000. Bound antibodies were detected using horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibodies (Amersham) in conjunction with the enhanced chemiluminescence detection system (ECL Plus; Amersham). For stripping and reprobing membranes were incubated for 30 min at 70°C in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris–HCl, pH 6.7, according to the manufacturer’s instructions (ECL Plus; Amersham).

Immunocytochemistry

For immunocytochemistry of tissue sections, 23-day-old and adult (more than 6-week-old) transgenic mice were used. Nontransgenic littermates were used as controls. Transgenic and nontransgenic mice were perfused with 4% paraformaldehyde in PBS (pH 7.4), brains were removed and postfixed in the same artificial cerebrospinal solution at 8–10°C, and cut into 150-μm-thick frontal-oriented slices using a Vibratome (FTB; Plano, Marburg, Germany). The corpus callosum was cut parallel to its axonal fascicles. Slices were transferred to a nylon net in bathing solution and kept at RT. For electrophysiological recordings, slices were placed in a chamber that was mounted on the stage of an upright confocal laser-scanning microscope (CLSM) (Sarastro 2000; Molecular Dynamics; Axiopt; Zeiss, Oberkochen, Germany). Brain slices were fixed within the chamber using a U-shaped platinum wire with a grid of nylon threads (Edwards et al., 1989). The chamber was continuously perfused with oxygenated bathing solution. Cell somata within the corpus callosum slices were visible in normal water immersion optics and could be approached by the patch electrode. GFP-expressing cells were selected under green epifluorescence (fluorescein filter set; Zeiss). Cell somata had a clear, dark membrane surface and were located 10–30 μm beneath the surface of the slice. Positive pressure was applied to the recording pipette while it was lowered to the slice under microscopic control. The cellular debris was blown aside and the tip could be placed onto the surface of the cell soma. Using the patch-clamp technique in whole-cell recording configuration, membrane currents were analyzed after activation by a series of depolarizing voltage steps (from −160 to +20 mV in 10-mV increments) (Hamill et al., 1981). Current signals were amplified with conventional electronics (EPC-7 amplifier; List Electronics, Darmstadt, Germany), filtered at 3 kHz, and sampled at 5 kHz by an interface (WinTida; HEKA, Lambrecht, Germany) connected to a personal computer system, which also served as a stimulus generator. After patch-clamp recording, cells were hyperpolarized to −150 mV to facilitate the dialysis of the fluorescent dye Lucifer yellow from the pipette solution into cellular processes. CLSM images were recorded before and after patch-clamp recording (laser excitation at 488 nm) and represent optical sections of 1 μm.

Solutions and electrodes. A standard bathing solution (134.0 mM NaCl, 2.5 mM KCl, 2.0 mM CaCl2, 1.3 mM MgCl2, 1.25 mM KH2PO4, 26.0 mM NaHCO3, 10.0 mM d-glucose, pH 7.4; with a total K+ concentration of 5 mM) was used. The solution was gassed with a mixture of 95% O2 and 5% CO2. The internal pipette solution had the following composition: 130 mM KCl, 0.5 mM CaCl2, 3.0 mM MgCl2, 5.0 mM EGTA, 3.0 mM Lucifer yellow, 10.0 mM Hepes, pH 7.2. Recordings were carried out at room temperature. Recording pipettes were made from boro Silicate capillaries (Hilgenreiner, Malsfeld, Germany). The open resistance of these patch pipettes was 4–5 MΩ.

Fluorescence-Activated Cell Sorting (FACS)

Brains and spinal cords were removed from 3- to 4-week-old transgenic PLP-GFP and wild-type mice. Meninges were removed by adhesion onto sterile Whatman No. 1 filter paper (Whatman, Hillsboro, OR). Tissues were minced into small pieces using a sterile razor blade and incubated for 30 min at 37°C in Hank’s balanced salt solution (HBSS) containing 1× pancreatin (Life Technologies) and 0.25% trypsin (Life Technologies). Subsequently,
tissue pieces were triturated gently in DMEM containing 10% FCS (DMEM/FCS). Cells were collected by centrifugation, incubated in the presence of DNase (0.5 mg/ml; Sigma) in HBSS for 5 min at 37°C, and again collected by centrifugation. For analytical flow cytometry, cells were resuspended in DMEM/FCS (0.5–1 × 10^6 cells/250 μl) and stained with antibodies to O4 (Sommer and Schachner, 1981) and phycoerythrin (PE)-coupled secondary antibodies (Vector Laboratories, Inc.). The percentage of double O4- and GFP-positive cells was determined by two-color analysis using a FACScan flow cytometer, equipped with an argon laser tuned at 488 nm, in combination with the CELLQuest software (Becton Dickinson, Bedford, MA). For detection of GFP-S65T and PE-derived fluorescence 530- and 585-nm band-pass filters were used, respectively. Debris was excluded from analysis by electronic gating.

For purification of mature oligodendrocytes, tissue homogenates were prepared as described above. Subsequently, cells were centrifuged at 1200 rpm in a tabletop centrifuge (Sorvall TC6; Sorvall, Inc., Newtown, CT) for 5 min and resuspended in PBS. The dissociated cells were mixed with 90% Percoll (Sigma) in a 3:2 ratio. Continuous density gradients were formed by centrifugation (SS34 rotor; Sorvall, Inc.; 10,000 rpm, 20 min). The upper myelin layer was removed, the intermediate layers containing oligodendroglial cells were collected, and the lower, erythrocyte-containing layer was discarded (Kim et al., 1984). After treatment with DNase, cells were resuspended in DMEM/25% FCS and GFP-expressing cells were purified using a FACS Vantage flow cytometer (Becton-Dickinson, Bedford, MA; supported through a gift from the W. M. Keck Foundation). Fluorescence emission was read with a 530/30-nm band-pass filter and debris was eliminated by adjusting the forward and side scatter. In a typical experiment, approximately 1 × 10^4 live GFP-positive cells per animal could be isolated for subsequent culture studies.

**Primary Cultures**

Cells purified by flow cytometry were plated onto poly-1-lysine (30 μg/ml; Sigma)-coated coverslips. Cells (1–2.5 × 10^4) were plated in 50-μl drops until cells had adhered to the coverslip (approximately 1 h). Cells were cultured in DMEM/10% FCS or DMEM containing 1× N2 supplement (Life Technologies) and 3.3 μM-triiodothyronine (T3) (10 ng/ml) (DMEM/N2/T3). In addition, cells were grown in either of the above media after conditioning by type I astrocytes. After 24 h, 50% volume of DMEM/N2/T3 was added to the astrocyte-conditioned medium to a final volume of 33% to ensure the presence of factors that are known to be essential for oligodendrocyte survival (Barres et al., 1993) and for morphological and functional maturation of postmitotic oligodendrocytes (Baas et al., 1997). Cells were analyzed under phase-contrast illumination and by immunocytochemistry.

Primary cultures of rat cortical type I astrocytes were obtained by the modified differential adhesion method (McCarthy and DeVellis, 1980; Stanimirovic et al., 1997). After reaching confluency, astrocytes were cultured for 2 to 3 days in DMEM/FCS or DMEM/N2/T3 to obtain astrocyte-conditioned medium. Astrocytes grown in DMEM/N2/T3 did not appear morphologically different from those grown in DMEM/FCS for at least 3 days in culture.

**RESULTS**

**Transgenic PLP-GFP Mice Were Generated That Express GFP mRNA and Protein in a Pattern Similar to Endogenous PLP**

In order to develop a novel tool that allows reliable analysis and isolation of in vivo-differentiated oligodendrocytes, we generated transgenic mice expressing GFP-S65T under the control of the PLP promoter (PLP-GFP mice). We chose the GFP-S65T mutant because of its red-shifted excitation maximum, which allows the use of standard FITC excitation–emission filter sets for analysis (Delagrange et al., 1995; Heim et al., 1995). For the generation of the transgenic construct we modified the one used by Wight et al. (1993) (Fig. 1). In the original PLP-LacZ construct, the sequence coding for β-galactosidase was inserted in frame with the sequence coding for the first 13 amino acids of PLP. Since β-galactosidase activity derived from this fusion protein was localized predominantly in the myelin, it was suggested that the N-terminal 13 amino acids of PLP were sufficient to target a protein to the myelin membrane (Wight et al., 1993). For translation/translocation of the transgenically expressed GFP to occur independently of PLP-derived amino acids, the ATG in exon 1 was mutated...
to GAG and the sequences coding for the first 13 amino acids of PLP were deleted, while keeping the exon–intron boundaries intact. The inclusion of intron 1 in the promoter construct appeared necessary since recent studies demonstrated the presence of regulatory elements within intron 1 (Wight and Dobretsova, 1997). After insertion of the sequences coding for the GFP-S65T variant, the Apa–SacII fragment of the PLP-GFP-S65T construct (Fig. 1) was used for injection into fertilized oocytes. Of 14 potential founder transgenic mice 1 male animal was proven positive for insertion of the transgene into the genome. This founder animal was used to generate a homozygous line, progeny of which were further analyzed. Homozygous animals showed no apparent pathological phenotype up to 9 months of age.

Northern blot analyses demonstrated that the developmental and tissue-specific expression of the transgene was consistent with that of the endogenous PLP gene (Fig. 2). No expression of GFP mRNA was detectable in wild-type animals (lane wt in Fig. 2Aa). Low levels of GFP and PLP mRNA were detected in brains of 1- and 5-day-old transgenic mice (lanes P1 and P5 in Figs. 2Aa and 2Ab). Expression of both GFP and PLP mRNAs showed a clear peak at postnatal day 21 (P21 in Figs. 2Aa and 2Ab) which corresponds to a peak in myelination. With age, however, there was a greater decrease in the level of GFP mRNA compared to PLP mRNA levels (compare lanes P32 in Figs. 2Aa and 2Ab). GFP mRNA was found predominantly in spinal cord and brain, with low levels detectable in testis (Fig. 2Ad). The same tissue-specific expression pattern was found for PLP mRNA, although the mRNA levels in spinal cord and brain were significantly higher for PLP than for GFP (compare exposure times for Figs. 2Aa and 2Ab or Figs. 2Ad and 2Ae). The size of the GFP mRNA was approximately 3.5 kb, whereas the predicted molecular weight of this mRNA was 1.1 kb. RT-PCR analyses of the coding region of the transgenic mRNA generated a fragment of the expected size, which was amplified using a primer pair specific for the PLP 5′-UTR and SV40 poly(A) sequences (not shown). Thus, there appeared to be a 3′ extension of the transgenic PLP-GFP mRNA beyond the SV40 poly(A) sequence present in the transgenic construct. We concluded that a poly(A) signal sequence derived from genomic sequences 3′ of the transgenic insertion site was used during transcription. In order to ensure that this mRNA alteration had no consequence on protein expression from the transgene, i.e., that correct GFP expression occurred in these mice, we performed Western blot analyses (Fig. 2B). In spinal cord, brain, and testis a protein with an apparent molecular weight of 27 kDa was detected (lanes spC, B, and Te in Fig. 2Ba). This size corresponds to the size of recombinant GFP-S65T protein (lanes GFP in Fig. 2Ba). Altogether, these data suggested that in the CNS the transgenic GFP is expressed by oligodendrocytes.
GFP Fluorescently Labels Oligodendrocytes, Which Can Be Visualized in Fixed and Live Tissue

For the visualization of GFP-positive cells in fixed and live tissue, we chose the following CNS areas, due to their relatively simple morphology: cerebellum, hippocampus, corpus callosum, and optic nerve. We did not perform a detailed analysis of GFP expression in the peripheral nervous system (PNS), since, despite the activity of the PLP promoter in the PNS (Wight et al., 1993), we could not directly detect cells fluorescently labeled by their GFP expression in teased fibers of the sciatic nerve. We, therefore, concluded that GFP expression levels in the PNS are too low to result in a directly detectable fluorescent label in situ in the transgenic PLP-GFP mice.

Analysis of Vibratome sections of adult cerebellum, using confocal imaging techniques, revealed GFP-positive cell bodies in the inner white matter tracts (Fig. 3). In addition, cells were detectable in a scattered distribution within the inner granular layer, but absent from the Purkinje cell and outer molecular layers. For a more accurate localization of oligodendrocytes and their myelin membranes, monoclonal antibodies specific for CNP, a myelin-associated enzyme (Braun et al., 1990), were used. As expected, cells positive for GFP could be identified in areas that were positive for CNP (Fig. 3, ++/+ CNP and ++/+ GFP). GFP-derived fluorescence was confined to cell bodies and nuclei. This predominant localization of GFP-derived fluorescence within cell bodies and nuclei appears to be a general feature of the unmodified GFP protein (Moriyoshi et al., 1996). However, a precise colocalization of cell bodies double positive for

FIG. 3. Analysis of GFP expression by confocal imaging using a 16× (top) or 63× (bottom) objective. Vibratome sections of homozgyous (+/+) and transgene-negative (−/−) adult cerebellum were viewed with laser excitation at 488 nm to visualize GFP (−/−, ++/+, and ++/+ GFP). In addition, oligodendrocytes and myelin were labeled using monoclonal antibodies specific for CNP in combination with Texas red-coupled secondary antibodies and laser excitation at 568 nm (+/+ CNP). GFP-positive cells were localized predominantly in white matter areas (wm), with scattered cells present in the inner granular layer (g). The Purkinje cell layer (p) and outer molecular layer (m) were devoid of GFP-positive cells. Arrows in lower left and right indicate an example of a typical CNP-positive oligodendrocyte that could be visualized by GFP-derived fluorescence in the cell body and nucleus. Scale bars: 50 μm, top; 10 μm, bottom.
GFP and CNP in myelin-rich CNS areas was not easily obtained, due to a more uniform myelin-associated CNP staining (Fig. 3, +/− CNP). Occasionally single cells could be distinguished at the edges of white matter tracts where the overall CNP stain was less, with clear GFP-positive cell bodies/nuclei and CNP-positive cytoplasm and processes (arrows in lower left and right of Fig. 3). To assess the identity of GFP-positive cells more accurately, we used antibodies that specifically label oligodendrocyte cell bodies (clone CC1) (Bhat et al., 1996), processes and somata of astrocytes (anti-GFAP), and neuronal nuclei (anti-NeuN) (Mullen et al., 1992) for double-labeling experiments (Fig. 4A). As a representative CNS region, we analyzed hippocampal areas, including the white matter tracts surrounding it (alveus and corpus callosum). These studies clearly demonstrated that neither astrocytes nor neurons were labeled by GFP (Fig. 4A, GFP/GFAP and GFP/NeuN).

In general, over 95% of the GFP-positive cells were also CC1 positive. In confocal overlays, however, some strongly GFP-labeled cells appeared CC1 negative, due to low levels of CC1 expression (not shown). Since CC1 expression in oligodendrocytes is not very well characterized for the different developmental stages of the oligodendrocyte lineage, the few CC1-negative, GFP-positive cells may represent an oligodendrocyte differentiation stage that is negative for the CC1 antigen, but positive for PLP-GFP expression. Additionally, CC1-positive, GFP-negative cells, particularly visible in areas with low density or apparent absence of oligodendrocyte cell bodies (cortical layers and stratum oriens of the hippocampal formation in Fig. 4A), most likely result from the weak staining by CC1 reported for subpopulations of astrocytes (Bhat et al., 1996).

The above data confirmed that GFP in the CNS of PLP-GFP mice is expressed exclusively by oligodendrocytes. However, although PLP is considered a myelin-specific protein (Yoshida and Colman, 1996), DM-20, an alternatively spliced product of the PLP gene, was shown to be expressed during embryonic development of the nervous system (Peyron et al., 1997; Timsit et al., 1992; Yu et al., 1994). Furthermore, detailed analysis of PLP and DM-20 expression at different stages of the oligodendrocyte lineage revealed that DM-20 is expressed in premyelinating oligodendrocytes, whereas PLP expression is specific for myelinating oligodendrocytes (Trapp et al., 1997). In order to evaluate whether in situ visualization of GFP-positive oligodendrocytes in the postnatal CNS of PLP-GFP mice is confined to mature oligodendrocytes or additionally allows the identification of DM-20-expressing, premyelinating oligodendrocytes, we performed immunocytochemical analyses using an antibody that recognizes an epitope unique to PLP (Asotra and Macklin, 1993). We analyzed cells at the leading edge of myelination in the cerebral cortex at postnatal day 14, because of the presence of a relative high number of premyelinating oligodendrocytes. We found that all GFP-positive cells in this area colocalize with PLP-immunoreactivity (Fig. 4B). These data clearly demonstrated that in the postnatal (14-day and older) CNS of the PLP-GFP mice GFP-derived fluorescence is directly detectable only in terminally differentiated and myelinating oligodendrocytes. The earliest stages of the oligodendrocyte lineage that, therefore, can be isolated and directly visualized by their GFP expression are cells that already started to myelinate axons. These results do not exclude GFP from occurring in premyelinating oligodendrocytes. However, levels of expression appear to be extremely low, and therefore, the presumably generated fluorescent label in these cells remains below detection limits for direct visualization in situ.

Overall, the above data demonstrated that GFP-positive, in vivo terminally differentiated oligodendrocytes could easily be visualized in fixed tissue. However, one of the main advantages in using GFP lies in the possibility of visualizing GFP-positive cells in live tissues. We studied the optic nerve in whole mount as a model to localize live GFP-positive cells. The optic nerve represents one of the simplest parts of the CNS, which contains primarily cell bodies of astrocytes, oligodendrocytes, and axons of retinal ganglion cells. During development, oligodendrocyte progenitor cells migrate into the nerve in a gradient from the chiasm to the retina. Subsequent differentiation occurs in a similar fashion (Colello et al., 1995). Consistent with this developmental program, GFP-positive cells could be detected only close to the chiasm in optic nerves dissected out of 6-day-old transgenic PLP-GFP mice, whereas in optic nerves of 32-day-old mice, GFP-positive cells were located over the whole length of the nerve (Fig. 5). In the most proximal (retinal) part of the optic nerve, where axons remain unmyelinated (Bartsch et al., 1994), there were no detectable GFP-positive cells even at postnatal day 32. These data demonstrated that GFP-positive oligodendrocytes can be visualized in live tissue from transgenic PLP-GFP mice. Furthermore, the restricted localization of GFP-positive cells in postnatal day 6 optic nerve, a developmental time point at which oligodendrocyte progenitor cells can be found throughout the length of the nerve, further confirmed that GFP expression in the PLP-GFP mice labels differentiated oligodendrocytes rather than oligodendrocyte precursor cells.

GFP-Positive Cells Express Symmetrical, Passive, Inward, and Outward Membrane Currents Characteristic of Mature Oligodendrocytes

Since the expression of specific ion channels by cells of the oligodendrocyte lineage correlates with differentiation stage, further evidence for the identity of the GFP-positive cells as mature oligodendrocytes could be obtained by electrophysiological methods. Brain slices from 7-week-old transgenic mice, which contained myelinated corpus callosum, were analyzed by the patch-clamp technique (Fig. 6). Cells were clamped at a potential of ±70 mV and membrane currents were activated by a series of de- and hyperpolarizing voltage steps ranging from −160 to +20 mV. All cells investigated (n = 6) displayed the same pattern of
symmetrical, passive, inward, and outward membrane currents carried by potassium (Fig. 6, right columns). Currents were slightly inactivating due to a shift in the Nernst potential during the voltage step. This type of current, also designated leak conductance, was shown previously to be characteristic of terminally differentiated, mature, O1 antigen-positive oligodendrocytes by in vitro (Barres, 1991; Sontheimer et al., 1989) and in situ investigations (Berger et al., 1991; Kirchhoff et al., 1997). In contrast, oligodendrocyte progenitor cells express predominantly voltage-gated, delayed outward-rectifying potassium currents. In order to determine whether GFP-positive cells in all brain areas display current characteristics of the more mature stages of the oligodendrocyte lineage, we analyzed the potassium current properties of GFP-positive cells in areas other than the corpus callosum, such as cortex, striatum, and hippocampus. In animals as young as postnatal day 9, all GFP-positive cells analyzed (n = 32) had lost mainly their voltage-gated, outward-rectifying potassium currents and displayed a symmetrical in- and outward potassium conductance (F. Kirchhoff and C. Schipke, unpublished observations). Therefore, we concluded that in the CNS of 9-day- to 7-week-old PLP-GFP mice GFP-positive cells display potassium current characteristics of mature oligodendrocytes.

Since GFP expression was visible only in cell bodies (Fig. 6, GFP, and Fig. 3), clamped cells were filled with the fluorescent dye Lucifer yellow to allow better morphological analysis of these cells. Using Lucifer yellow it was previously shown that small numbers of oligodendrocytes are dye coupled and that dye coupling occurs at contacts between adjacent cell bodies (Butt and Ransom, 1993). In our experiments, we only rarely observed pairs of adjacent

FIG. 4. Visualization of GFP-positive cells (GFP in A and B) and GFP-CC1 (GFP/CC1 in A), -GFAP (GFP/GFAP in A), -NeuN (GFP/NeuN in A), and -PLP (PLP in B) double-positive cells in Vibratome sections of brains from 23-day-old (in A) and 14-day-old (in B) transgenic PLP-GFP mice using confocal imaging techniques. In (A), a region of the white matter tract (wm) surrounding the hippocampus is shown (p, pyramidal cells of the hippocampal formation; so, stratum oriens of the hippocampal formation; c, cortical layers). Only CC1-GFP double-positive cells could be detected. No GFP-GFAP or GFP-NeuN double-positive cells were seen, indicating that GFP specifically labels oligodendrocytes. In (B), a typical area at the leading edge of myelination in the cortex of a 14-day-old PLP-GFP mouse is shown. Sections were immunostained with PLP-specific antibodies in combination with Texas red-coupled secondary antibodies. The same section is displayed on the left with laser excitation at 488 nm (GFP) and on the right with laser excitation at 568 nm (PLP). All GFP-positive cells analyzed were also positive for PLP. Arrows on the left (GFP) and right (PLP) indicate two typical GFP/PLP double-positive cells that are located in an area that contains a significant number of DM20-positive, but PLP-negative, premyelinating oligodendrocytes (see Trapp et al., 1997). Scale bars, 100 μm.

FIG. 5. Visualization of GFP-positive cells in whole mounts of optic nerves from 6-day-old (P6) and 32-day-old (P32) transgenic PLP-GFP mice. Nerves were analyzed using a photomicroscope. Optic nerves were oriented with the end close to chiasm on the left and the end close to the retina on the right. Scale bar, 100 μm.
cell bodies that were coupled. However, since we were focusing on the display of the morphological characteristics of the GFP-positive oligodendrocytes, we did not optimize our experimental conditions for the display of gap junctions. For all cells analyzed, parallel-oriented processes, which are typical of axon-ensheathing oligodendrocytes, became clearly visible with Lucifer yellow. These data further confirmed the differentiated and myelinating stage of the GFP-positive oligodendrocytes (Fig. 6, LY). No astrocytes, which are characterized by numerous fine, highly branched, irregularly oriented processes, were patched although they are present at a similar density (Fig. 4, GFP/GFAP). Most importantly, these initial analyses clearly demonstrated that brain slices obtained from transgenic PLP-GFP mice are ideally suited for comprehensive physiological characterization of differentiated oligodendrocytes in situ.

**GFP-Positive Oligodendrocytes in Culture Possess a Mature Phenotype**

After establishing that GFP expression in the postnatal (9-day- to 7-week-old) CNS of transgenic PLP-GFP mice is restricted to mature oligodendrocytes, we developed a protocol for isolation and culture of in vivo-differentiated oligodendrocytes for further analysis of their functional properties. First, we scanned enzymatically dissociated cells from spinal cords (Fig. 7) and brains (not shown) of 3- to 4-week-old transgenic and wild-type mice by analytical flow cytometry. For better analysis, debris and erythrocytes were excluded by adjusting forward and side light scatter gates. In order to confirm the identity of sorted GFP-positive cells as oligodendrocytes, cell suspensions were immunostained with the monoclonal antibody O4. The O4 antibody recognizes a cell surface antigen that appears at an intermediate stage of oligodendroglial development, when the cell is fully committed to terminal oligodendrocyte differentiation, and that remains present when the cells mature (Pfeiffer et al., 1993; Warrington and Pfeiffer, 1992). Approximately 30% of the cells analyzed were O4-positive in cell suspensions derived from spinal cords, whereas preparations from brain contained only approximately 10% O4-positive cells in cell populations analyzed under similar conditions (data not shown). In cell preparations from transgenic spinal cords, approximately two thirds of the O4-positive cells were also GFP-positive and almost all of them were coupled. However, since we were focusing on the display of the morphological characteristics of the GFP-positive oligodendrocytes, we did not optimize our experimental conditions for the display of gap junctions. For all cells analyzed, parallel-oriented processes, which are typical of axon-ensheathing oligodendrocytes, became clearly visible with Lucifer yellow. These data further confirmed the differentiated and myelinating stage of the GFP-positive oligodendrocytes (Fig. 6, LY). No astrocytes, which are characterized by numerous fine, highly branched, irregularly oriented processes, were patched although they are present at a similar density (Fig. 4, GFP/GFAP). Most importantly, these initial analyses clearly demonstrated that brain slices obtained from transgenic PLP-GFP mice are ideally suited for comprehensive physiological characterization of differentiated oligodendrocytes in situ.

**FIG. 6.** Patch-clamp analysis of GFP-positive cells in corpus callosum slices. 150-μm slices were obtained from 7-week-old transgenic PLP-GFP mice and whole-cell membrane currents were investigated. The top and bottom are recordings from two representative cells. GFP-positive cells selected for patch-clamp analysis are marked with white arrows (GFP). Dialysis of the selected cells with Lucifer yellow (LY) visualized parallel-oriented processes that are characteristic of mature oligodendrocytes after myelination of axonal processes. Activation of membrane currents by a series of de- and hyperpolarizing voltage steps (−160 to +20 mV) revealed symmetrical, passive, inward, and outward currents dominating the membrane conductance (right column). Scale bars, 10 μm.
the GFP-positive cells were O4-positive (Fig. 7, right plot). These data suggested that fluorescence-activated cell sorting is likely to yield highly pure cell preparations of in vivo-differentiated oligodendrocytes from cell suspensions derived from the CNS of 3- to 4-week-old transgenic PLP-GFP mice. The appearance of approximately 4.5% GFP-positive/O4-negative cells (Fig. 7, right plot, lower right quadrant) may result from a loss of the O4 surface antigen during cell dissociation or it might reflect oligodendrocyte heterogeneity, the existence of which has been suggested previously (Fanarraga and Milward, 1997).

In order to purify GFP-positive cells in larger quantity, we used combined suspensions of spinal cord and brain cells for fluorescence-activated cell sorting. Prior to sorting, erythrocytes and debris were removed from the cell suspensions by centrifugation through a density gradient. In order to obtain highly pure populations of GFP-positive oligodendrocytes, cells were considered positive only if their fluorescence was brighter than 99% of control cells, which were derived from the CNS of age- and strain-matched nontransgenic mice. By these criteria approximately 5% of the live cells were isolated as GFP-positive in vivo-differentiated oligodendrocytes (Fig. 8A). When these cells were cultured in defined medium or in medium containing FCS, all of the surviving cells were GFP and GC positive after 48 h in culture. These cells, however, remained round in morphology with no obvious processes (data not shown). Since it has been shown that survival and differentiation of oligodendrocyte progenitor cells is regulated by astrocyte-derived factors (Barres and Raff, 1994), we cultured the purified GFP-positive cells in the presence of astrocyte-conditioned medium. After 48 h, approximately 10% of the surviving cells began to develop a more complicated morphology with multiple processes and myelin sheath-like structures (Fig. 8B), a phenotype often described for mature oligodendrocytes in culture (Pfeiffer et al., 1993). In conclusion, these data indicate that the culture system described above provides a suitable tool for the characterization of yet unidentified factors which may be required for survival/ maturation of in vivo terminally differentiated oligodendrocytes.

**DISCUSSION**

The current studies describe the generation and characterization of transgenic mice that express GFP under the control of the PLP promoter. In the CNS of these mice, cells fluorescently labeled by their GFP expression could be clearly identified as mature oligodendrocytes by immunocytochemical studies, by the spatiotemporal expression pattern of the transgene in the optic nerve, and by electrophysiological potassium current analysis of corpus callosum slices. Furthermore, we demonstrated that differentiated oligodendrocytes could easily be visualized in live, as well as fixed, tissue, both from actively myelinating and from adult (7-week-old) animals. This convenient localization provided an expedient approach to electrophysiological characterization of highly differentiated oligodendrocytes in situ. In addition, we introduced a novel purification and tissue culture system for in vivo-differentiated oligodendrocytes that now permits a detailed analysis of their molecular and cell biological features.

In the PLP-GFP mice, developmental and tissue-specific expression of the transgenic GFP mRNA appears to be entirely regulated by the PLP-derived promoter sequences.
explain the continued high levels of endogenous PLP stabilizing elements in the 3 levels remain high. The presence of putative mRNA-day 25 (Macklin transcription rate of the PLP gene decreases after postnatal lines of PLP-LacZ mice (Wight described here, but was also found for three independent promoter-driven mRNA is not unique to the transgenic line indicating robust protein expression. The discrepancy between levels of endogenous PLP mRNA, and, in contrast to the PLP mRNA, a decrease in GFP mRNA from postnatal day 21 to 32 was noticeable. Despite this reduction in mRNA levels, however, differentiated oligodendrocytes could easily be identified in the adult CNS by their GFP-derived fluorescence, indicating robust protein expression. The discrepancy between levels of endogenous PLP mRNA and transgenic, PLP promoter-driven mRNA is not unique to the transgenic line described here, but was also found for three independent lines of PLP-LacZ mice (Wight et al., 1993). In the CNS, the transcription rate of the PLP gene decreases after postnatal day 25 (Macklin et al., 1991), while endogenous PLP mRNA levels remain high. The presence of putative mRNA-stabilizing elements in the 3' untranslated region may explain the continued high levels of endogenous PLP mRNA in the adult CNS (Wight et al., 1993; B. Mallon and W. B. Macklin, unpublished observations). Consequently, without the PLP-specific 3' sequences, transgenic GFP mRNA levels may more closely correlate with the transcription rate of the PLP gene and decrease with lower rates of transcription. In addition, in the transgenic PLP-GFP mice, a genomic poly(A) signal, derived from the insertion site, seems to be utilized for GFP termination, which could contain mRNA-destabilizing elements. Nonetheless, the developmental and tissue-specific expression of the transgenic GFP mRNA appears to be entirely regulated by PLP-derived sequences. These data are consistent with recent findings that despite the random insertion of transgenes into the genomic DNA, local effects appear to have minimal impact on the transgene's expression in those cells that initiate transcription (Graubert et al., 1998). Furthermore, the absence of any obvious pathology in the transgenic PLP-GFP mice indicates that transgene integration does not interfere with the expression of endogenously expressed genes.

While the studies described here were focused on the expression of GFP in the CNS, it is noteworthy to mention that PLP-driven GFP mRNA and endogenously expressed PLP mRNA were additionally detected in the testis. Similarly, testis expression was demonstrated for the oligodendrocyte-derived proteins 2',3'-cyclic nucleotide 3'-phosphodiesterase (Gravel et al., 1998) and oligodendrocyte-specific protein (OSP/Claudin-11; Morita et al., 1999). This shared expression of certain proteins might be attributed to structural similarities between oligodendrocytes in the CNS and Sertoli cells in the testis, as was recently suggested by Morita et al. (1999).

The presence of in situ-labeled mature oligodendrocytes allowed us for the first time to distinguish unequivocally in vivo-differentiated oligodendrocytes in the postnatal CNS from other neighboring glial cells such as astrocytes and progenitor cells prior to patch-clamp analyses. Therefore, we were able to record current characteristics from highly differentiated oligodendrocytes such as those present in the corpus callosum of 7-week-old mice. Studies like this are of particular advantage for a comparative analysis of oligodendrocyte physiology in gray and white matter, to assess, for example, whether oligodendrocytes that reside in gray matter areas might represent a subpopulation of oligodendroglial cells with novel, region-specific electrophysiological properties. The usefulness of cell-specific GFP expression for electrophysiological analyses is supported by recent studies identifying genetically modified DRG neurons after transfection with an adenovirus construct and demonstrating that the GFP label does not change the electrophysiological properties of the transfected cells (Smith et al., 1997). Thus, the PLP-GFP mice will likely facilitate many physiological studies of differentiated oligodendrocytes in situ.

To permit more detailed cell biological analyses of in vivo-differentiated oligodendrocytes under easily manipulable conditions, we developed a protocol for isolation and

**FIG. 8.** Purification and culture of GFP-positive oligodendrocytes by flow cytometry. The single-parameter fluorescence histogram in (A) shows the distribution of live cells according to their fluorescence intensity for GFP (x axis, logarithmic scale). Cell numbers are plotted on the y-axis. The distribution of cells derived from spinal cords and brains of transgenic PLP-GFP mice is delineated as a solid line and from control animals as a dotted line. Cells within the area marked by the bar were considered GFP positive (5% of total live cells) and collected. Purified oligodendrocytes in culture developed an appearance of myelinogenic oligodendrocytes 48 h after plating only in the presence of astrocyte-conditioned medium. Without the astrocyte conditioned medium, attached cells remained round without processes. In (B) the same field is shown under laser excitation at 488 nm (for GFP) and at 568 nm (for GC/Texas red). All surviving cells were GFP and GC positive. Cells portrayed here were grown in astrocyte-conditioned DMEM/FCS that was supplemented with DMEM/N2/T3 24 h after plating.
culture of in vivo-differentiated, GFP-expressing oligodendrocytes. In order to estimate the percentage of GFP-positive cells that were isolated by this procedure, we referred to the following observations. The total number of O4-positive oligodendrocytes in the CNS was shown to increase from birth to postnatal day 5 when it reaches a plateau (Gard and Pfeiffer, 1989). For the telencephalon of a 5-day-old rat the presence of $1.5 \times 10^5$ O4-positive oligodendrocytes was estimated (Gard and Pfeiffer, 1989), while for a 7-day-old mouse cerebellum $1.5 \times 10^6$ O4-positive cells were calculated (Meier et al., 1982). Furthermore, in our experiments approximately 65% of O4-positive oligodendrocytes appeared to be GFP positive. However, previously described data, using O4-positive cells from postnatal day 13 rat cerebrum, indicated that only 40% of the O4-positive cells represent mature oligodendrocytes (Shinar and McMorris, 1995). The increased percentage of O4-positive cells that would be defined as mature by their GFP expression in our studies appears reasonable, since CNS tissue from older animals, including spinal cord, was used. Therefore, assuming that a 3- to 4-week-old mouse CNS contains approximately $1.5 \times 10^5$ O4-positive oligodendrocytes, of which two-thirds are GFP positive, a recovery of approximately 1% was achieved by the isolation of approximately $1 \times 10^4$ live GFP-positive cells per animal. Yields this low have been described previously for the isolation of differentiating oligodendrocytes from animals beyond postnatal day 5 (Gard and Pfeiffer, 1989) and are likely to reflect the difficulties in isolating living oligodendrocytes that have already established a complex morphology. Higher yields were described for the isolation from spinal cord (Wood and Bunge, 1991), which is consistent with our observations. Given the estimated low recovery rates, however, we cannot exclude that, under the conditions used, preferentially a certain subpopulation of in vivo-differentiated oligodendrocytes was isolated. Nevertheless and more importantly, the culture system introduced here provides a unique and novel system for a detailed analysis of the environmental requirements for survival and maturation of oligodendrocytes that developed in situ to a stage at which they are terminally differentiated and begin to myelinate axons.

Oligodendrocyte development and myelination are regulated partially by intrinsic mechanisms, but are also significantly influenced by signals derived from other cell types, such as astrocytes and neurons (Barres and Raff, 1994). In this context, it is noteworthy that after isolation by flow cytometry, the ability to culture GFP-positive oligodendrocytes with a process-bearing, mature phenotype was critically dependent upon factors released by astrocytes. In other studies, it has been demonstrated that survival of cells at the different stages of oligodendrocyte differentiation requires different growth factors. For example, combinations of known astrocyte-derived molecules are apparently sufficient for survival of oligodendrocyte progenitor cells (Barres et al., 1993), whereas for survival of immunopanned, newly differentiated oligodendrocytes, an additional leukemia inhibitory factor-like protein appears to be essential (Gard et al., 1995). Therefore, differentiated oligodendrocytes of the mature CNS, such as the GFP-positive cells used in our studies, may need yet unidentified environmental factors for survival and/or for the maintenance of a myelinogenic state. Our data focus primarily on the requirement for astrocyte-derived factors in order for mature cells to reestablish a process-bearing phenotype in culture. Thus, our data may suggest that astrocyte-derived signals might be involved in the formation of myelin sheath-like structures in vitro and possibly, in addition to neuron-derived components (Barres and Raff, 1993; Whittemore et al., 1993), in the formation of the myelin sheath itself. The importance of an interaction between oligodendrocytes and astrocytes for the formation of the myelin sheath is supported by data obtained in model systems of remyelination and injury (Blakemore, 1984; Franklin et al., 1991). Furthermore, a link between astrocyte function and the maintenance of the myelin sheath is suggested by a late-onset defect of myelination homeostasis in GFAP−/− animals (Liedtke et al., 1996). However, it remains to be elucidated whether, and which, soluble astrocyte-derived factors regulate survival and/or the myelinogenic capacity of differentiated oligodendrocytes.

For the most part, current literature about the functional regulation of differentiated oligodendrocytes remains confusing. Much of the data focus on myelin gene expression by neonatal oligodendrocytes in culture, and integration of the published data on differentiating oligodendrocytes is complicated by differences in species, culture conditions, and antibody specificities. Furthermore, it cannot be excluded that remyelination by cells that have undergone myelination in situ requires different environmental clues than myelination during development. For example, Ludwig and Szuchet (1993) demonstrated that adult ovine oligodendrocytes in culture do not myelinate dorsal root ganglion neurons, whereas neonatal rodent oligodendrocytes do (Wood and Bunge, 1991). Additionally, Ladiwala et al. (1998) demonstrated that NGF does not induce apoptosis in adult human oligodendrocytes, whereas neonatal rodent oligodendrocytes were shown to undergo NGF-induced apoptosis (Casaccia-Bonnefil et al., 1996).

Thus, an evaluation of the functional properties of mature oligodendrocytes in situ and of their myelinating/remyelinating capabilities could provide clarifying data about the different mechanisms that may be involved in the formation of the myelin sheath during normal development and in remyelination of demyelinated axons under pathological conditions. Analyses such as these have the potential to vastly improve strategies for enhanced remyelination during CNS repair.

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