Generation and Characterization of Brain Lipid-Binding Protein Promoter-Based Transgenic Mouse Models for the Study of Radial Glia

RALF S. SCHMID, YUKAKO YOKOTA, AND E. S. ANTON*  
Neuroscience Center and Department of Cell and Molecular Physiology, University of North Carolina School of Medicine, Chapel Hill, North Carolina

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ABSTRACT
Radial glia play an essential role in the generation of the cerebral cortex through their function as neuronal precursors and as neuronal migration guides. A molecular marker for radial glia in the developing central nervous system is the brain lipid-binding protein (BLBP). To generate mouse models for the visualization and study of radial glia, we expressed EGFP, EYFP, or dsRed2 in transgenic mice under the control of the BLBP promoter. In these transgenic lines, fluorescent protein expression is restricted to radial glia in the embryonic cortex and to astrocytes in the adult brain. Electroporation of the transgenes into embryonic cortex also resulted in radial glia-specific transgene expression. These BLBP promoter driven transgenic mice and organotypic models for the visualization and study of radial glia, we postulated neuronal precur- 
sors and as a scaffold for organizing neuronal migration and placement in the developing cerebral cortex (Miyata et al., 2001; Noctor et al., 2002; Malatesta et al., 2000, 2003). Around the time of birth, as neuronal generation and migration dwindle, radial glia in the cerebral wall transform into astrocytes or intermediate neuronal precursors (Schmechel and Rakic, 1979; Hunter and Hatten, 1995; Noctor et al., 2004). Radial glia are not limited to the developing cerebral wall since analogous radial glia-like cells are present outside of the cerebral cortex as well. These include radial glia in the spinal cord, Bergmann glia in the cerebellum, and Müller glia in the retina. Both Bergmann glia and Müller glia morphologically persist in the adult nervous system. As in the cerebral cortex, these radial glia-like cells contribute to the generation of the characteristic neuronal organization in their respective CNS regions. Radial glia are often defined by their distinct morphology: bipolar cells with the soma located near the ventricular surface, and elongated processes spanning the entire width of the cerebral wall (Rakic, 1971, 1972). A variety of cell type-specific molecular markers can also be used to delineate radial glia in the developing cerebral cortex. For example, nestin and the antigen for the monoclonal antibodies RC1 and RC2 are expressed in radial glia as early as embryonic day 9.5 (E9.5) (Misson et al., 1988; Chanas-Sacre, 2000). Around E13, a second set of molecular markers is expressed selectively in radial glia: brain lipid-binding protein (BLBP), glutamate transporter (GLAST), and Tenascin C (Hartfuss, 2001; Campbell and Gotz, 2002; Noctor et al., 2001, 2004). Expression of this latter set of molecular markers persists after the transformation of radial glia into astroglia. Immunolabeling of the embryonic cerebral cortex with antibodies to these markers, as well as the expression profiles of other signaling molecules, such as ephrin B1 and extra-large G protein X1as, indicates nonoverlapping patterns of expression in radial glia, thus suggesting the existence of distinct subpopulations of radial glial cells (Kehlenbach, 1994; Stuckmann, 2001; Kriegstein and Gotz, 2003; Malatesta et al., 2003). Recent studies, however, also raise the possibility that all radial glia undergo similar patterns of gene expression during development and that distinct antigenic profiles of radial glia reflect the particular developmental stage the respective radial glia subpopulation is in (Anthony et al., 2004). To generate tools for the analysis of the fate and function of radial glial cell populations in the developing cerebral cortex, we sought to establish transgenic mice lines in which radial glia from different brain regions can be readily identified with different fluorescent markers. Recently, the promoter region of the radial glia-specific marker BLBP has been defined and a minimal promoter sequence necessary for specific expression in radial glia from E13 in the developing CNS has been characterized (Feng and Heintz, 1995; Anthony et al., 2004). We used this minimal

INTRODUCTION
Radial glia play an essential role in the emergence of the laminar organization of the neurons in the cerebral cortex (Rakic, 1971, 1972). They serve as neuronal progenitors and as a scaffold for organizing neuronal migration and placement in the developing cerebral cortex (Miyata et al., 2001; Noctor et al., 2001; Hatten, 2002; Malatesta et al., 2000, 2003). Around the time of birth, as neuronal generation and migration dwindle, radial glia in the cerebral wall transform into astrocytes or intermediate neuronal precursors (Schmechel and Rakic, 1979; Hunter and Hatten, 1995; Noctor et al., 2004). Radial glia are not limited to the developing cerebral wall since analogous radial glia-like cells are present outside of the cerebral cortex as well. These include radial glia in the spinal cord, Bergmann glia in the cerebellum, and Müller glia in the retina. Both Bergmann glia and Müller glia morphologically persist in the adult nervous system. As in the cerebral cortex, these radial glia-like cells contribute to the generation of the characteristic neuronal organization in their respective CNS regions. Radial glia are often defined by their distinct morphology: bipolar cells with the soma located near the ventricular surface, and elongated processes spanning the entire width of the cerebral wall (Rakic, 1971, 1972). A variety of cell type-specific molecular markers can also be used to delineate radial glia in the developing cerebral cortex. For example, nestin and the antigen for the monoclonal antibodies RC1 and RC2 are expressed in radial glia as early as embryonic day 9.5 (E9.5) (Misson et al., 1988; Chanas-Sacre, 2000). Around E13, a second set of molecular markers is expressed selectively in radial glia: brain lipid-binding protein (BLBP), glutamate transporter (GLAST), and Tenascin C (Hartfuss, 2001; Campbell and Gotz, 2002; Noctor et al., 2001, 2004). Expression of this latter set of molecular markers persists after the transformation of radial glia into astroglia. Immunolabeling of the embryonic cerebral cortex with antibodies to these markers, as well as the expression profiles of other signaling molecules, such as ephrin B1 and extra-large G protein X1as, indicates nonoverlapping patterns of expression in radial glia, thus suggesting the existence of distinct subpopulations of radial glial cells (Kehlenbach, 1994; Stuckmann, 2001; Kriegstein and Gotz, 2003; Malatesta et al., 2003). Recent studies, however, also raise the possibility that all radial glia undergo similar patterns of gene expression during development and that distinct antigenic profiles of radial glia reflect the particular developmental stage the respective radial glia subpopulation is in (Anthony et al., 2004).

To generate tools for the analysis of the fate and function of radial glial cell populations in the developing cerebral cortex, we sought to establish transgenic mice lines in which radial glia from different brain regions can be readily identified with different fluorescent markers. Recently, the promoter region of the radial glia-specific marker BLBP has been defined and a minimal promoter sequence necessary for specific expression in radial glia from E13 in the developing CNS has been characterized (Feng and Heintz, 1995; Anthony et al., 2004). We used this minimal
BLBP promoter sequence to drive EGFP, EYFP, or dsRed2 expression, and we generated several lines of transgenic mice (referred to as BLBP-EGFP, EYFP, dsRed2 mice). Analysis of different regions of the embryonic and mature brain from these mice indicates that the fluorescent proteins were distinctly expressed in radial glia and astrocytes of the cerebral cortex, cerebellum, and hippocampus. Neuronal expression of the fluorescent markers is not evident. These BLBP-EGFP, EYFP, and dsRed2 transgenic mouse lines and transgene expression in organotypic brain slices can be useful tools in the evaluation and delineation of the mechanisms underlying the development and differentiation of radial glia and astrocytes.

**MATERIALS AND METHODS**

**Generation of BLBP-EGFP, EYFP, dsRed2 Transgenic Mice**

The BLBP-EGFP and EYFP transgene was constructed by removing the BLBP promoter coding region from the BLBP promoter-LacZ pCAT3 reporter plasmid (gift of Dr. N. Heintz, Rockefeller University, New York) by SacII and XbaI digestion, followed by ligation with EGFP or EYFP fragments from pEGFP-N1 or pEYFP-N1 plasmids (Clontech, Mountain View, CA). The BLBP promoter used is the minimal promoter region essential for radial glia-specific expression of BLBP in the cerebral cortex (Feng and Heintz, 1995). To generate the BLBP-dsRed2 transgene, the same strategy was used, except that the BLBP promoter was removed by NotI and XbaI digestion before ligation with the dsRed2 fragment from the pdsRed2-N1 plasmid (Clontech). The identity of the BLBP-EGFP, EYFP, or dsRed2 transgene was verified by restriction digest and sequencing. To generate transgenic mice, the injection fragment was excised from the BLBP-EGFP, EYFP, or dsRed2 plasmid with ClaI, separated by agarose gel electrophoresis, purified, and used for pronuclear microinjection of fertilized eggs from FVB/N strain mice. Polymerase chain reaction (PCR) analysis and Southern blotting characterization of transgenic lines are described in supplemental information.

**BLBP-EGFP, EYFP, or dsRed2 Expression Analysis In Vitro**

Transgene expression was analyzed in transfected C6R glioma cells, COS7 cells, HEK293 cells, and primary embryonic cortical cells. See supplemental information for details.

**Analysis of Transgene Expression in BLBP-EGFP, EYFP, or dsRed2 Transgenic Mice**

Transgene expression was analyzed in the brains of embryonic or adult BLBP-EGFP, EYFP, or dsRed2 mice. See supplemental information for details.

**Electroporation and Slice Assays for Radial Glial Visualization**

E16 mouse embryonic cortices were electroporated with BLBP-EGFP, EYFP, or dsRed2 cDNA and analyzed as described in the supplemental information.

**RESULTS**

**Generation of Transgenic Mice Expressing Fluorescent Protein in Radial Glia**

A transgene encoding EGFP, EYFP, or dsRed2 under the control of the BLBP promoter was constructed (Fig. 1). Previous studies indicate that this promoter can drive gene expression specifically in radial glia as early as E12.5 (Anthony et al., 2004; Feng and Heintz, 1995). We verified the radial glia-specific expression of these transgenes first in the radial glial cell line C6R (B–D), or control COS7 cells (E–G). After 24–36 h, EGFP, EYFP, or dsRed2 expression was detected only in C6R cells (B–D), but not in COS7 cells (E–G). COS7 cells transfected with pCMV-EGFP, EYFP, or dsRed2 express the respective fluorescent protein (E–G, inset). Cell nuclei were counterstained with bis-benzimide (blue). Scale bar = 20 μm.

**Fig. 1.** BLBP promoter driven expression of EGFP, EYFP, or dsRed2 in radial glia in vitro. A: Transgene construct used for the generation of BLBP promoter-EGFP, EYFP, or dsRed2 transgenic mice. Relevant enzymatic restriction sites are indicated. B–G: BLBP-EGFP, EYFP, or dsRed2 were transfected into the radial glial cell line C6R (B–D), or control COS7 cells (E–G). After 24–36 h, EGFP, EYFP, or dsRed2 expression was detected only in C6R cells (B–D), but not in COS7 cells (E–G). COS7 cells transfected with pCMV-EGFP, EYFP, or dsRed2 express the respective fluorescent protein (E–G, inset). Cell nuclei were counterstained with bis-benzimide (blue). Scale bar = 20 μm.
antibodies (Tuj1; Fig. 2). EGFP, EYFP, or dsRed2 expression was only evident in RC2\(^+\) radial glia cells, but not in Tuj1\(^-\) neurons. In contrast, both neurons and radial glia expressed fluorescent proteins when transfected with CMV promoter-EGFP, EYFP, or dsRed2 plasmids (data not shown). We next ventricle injected and electroporated E16 mouse cortices with BLBP-EGFP, EYFP, or dsRed2 plasmids. Cortical slices from electroporated brains were imaged after 24 h in vitro. A large selection of radial glial cells in the embryonic cortical slices was found to express fluorescent proteins (Fig. 3). The radial glial cell soma in the ventricular or subventricular zone (VZ/SVZ), the corresponding radial glial processes spanning the entire width of the cortex, and the radial glial endfeet are delineated by strong fluorescent protein expression. Migrating neurons appear not to express fluorescent proteins. These results demonstrate that the BLBP promoter specifically drives the expression of EGFP, EYFP, or dsRed2 in embryonic radial glial cells.

To express fluorescence proteins selectively in radial glial cells of the developing CNS, transgenic mice expressing EGFP, EYFP, or dsRed2 under control of the BLBP promoter were generated. A total of 41 founder lines of BLBP-EGFP mice, 22 of BLBP-EYFP mice, and 17 of BLBP-dsRed2 mice were identified by PCR genotyping of tail DNA, and verified by Southern blot analysis of tail DNA with a probe specific for the respective transgene (Fig. 4). Independent breeding lines were established from at least three different founders for each transgene (named BG-1-3, BY1-3, BR-1-3, corresponding to the transgene BLBP-EGFP, BLBP-EYFP, or BLBP-dsRed2, respectively).

We first analyzed transgene expression in E14 cerebral cortices since our in vitro studies and previous characterization of the BLBP promoter suggested initial expression of the BLBP promoter driven transgenes at this developmental stage (Feng and Heintz, 1995). EGFP, EYFP, or dsRed2 was expressed prominently in radial glia cell soma and moderately in radial glial processes.
By E16, strong expression of fluorescent protein was found throughout the developing cerebral wall in many cells with long thin processes and their soma in the ventricular or subventricular zone (Fig. 5C–J). Similar expression of fluorescent protein in radial glia was found in E18 cerebral cortices as well (Fig. 5K,L). The transgene expressing cells were RC2\(^*\), confirming the radial glial identity of these cells. Distinct differences in transgene expression within subsets of radial glia in the E16 cerebral wall were not detected and Tuj1\(^*\) neuronal cells did not detectably express the transgene. As noted previously with this promoter (Feng and Heintz, 1995), no expression of fluorescent protein was detected in the E16 spinal cord. During the early postnatal days, radial glia transform into astrocytes and the BLBP promoter remains active in astrocytes. Consistent with this, we observed transgene expression in GFAP\(^+\) astrocytes of the adult cerebral cortex and hippocampus (Figs. 6 and 7). The subgranular zone astroglial cells of the dentate gyrus, a known source of neural stem cells in mature hippocampus,

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**Fig. 4.** Generation and analysis of BLBP-EGFP, EYFP, or dsRed2 transgenic mice. **A:** Southern blot analysis of founder mice. Three BLBP-EGFP (lanes 1–3) and three BLBP-EYFP (lanes 4–6) transgenic mouse lines show transgene expression when tail DNA was hybridized with a probe specific for EGFP (lanes 1–3) or EYFP (lanes 4–6). No transgene was detected in wildtype mice (wt lane). A probe specific for Sox2 was used as loading control. Size markers are indicated on the right. **B:** PCR genotyping of transgenic mouse lines. Primers specific for BLBP-EGFP/EYFP or BLBP-dsRed2 were used. A single PCR product of 420-bp (EGFP/EYFP) or 450-bp (dsRed2) size was detected for the respective transgenic mouse lines (lanes 1–10). As positive control (+ lane), transgene plasmids (BLBP-EGFP, EYFP, or dsRed2) were used in the PCR reaction. DNA from wildtype mice (wt lane) or no DNA (– lane) was used as negative controls. Size markers are indicated on the left.

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**Fig. 5.** Expression of fluorescent proteins in the embryonic cerebral cortex of BLBP-EGFP, EYFP, or dsRed2 transgenic mice. Representative images from transgenic lines throughout development. **A,B:** Images of E14 cerebral wall from BLBP-EGFP, or dsRed2 transgenic mice. Distinct EGFP (A) or ds-Red2 (B) expression in radial glial soma in the ventricular zone of the cerebral wall is evident (arrowheads, A,B). **C–J:** Images of E16 cerebral wall from the BLBP-EGFP, EYFP, or dsRed2 transgenic mice. ds-Red2 (C) or EYFP (D) is expressed in radial glial processes spanning the cerebral wall (arrowheads, C,D). **E–J:** Expression of fluorescent protein is specific to radial glia. EGFP expressing radial glial (arrowhead, E,H) from two different BLBP-EGFP transgenic mice (BG-1, BG-2) co-label with radial glial-specific RC2 antibodies (arrowhead, F,I). The merged images (G,J) demonstrate the radial glial identity of the fluorescent protein expressing cells. **K,L:** In E18 cerebral wall, as at E16, radial glial soma and processes (arrowheads) express EGFP (K) or EYFP (L). EYFP expression in D was pseudo-colored to yellow using Zeiss Pascal image analysis software. A,E–G,K, Line BG-1; B,C, Line BR-1; D,K, Line BY-1. P, pial surface; V, ventricular surface. Scale bars = 35 μm in A,C; 20 μm in B,D–L.
**Discussion**

Radial glia act as guides for neuronal migration and as a source of neuronal precursors during the development...
of the cerebral cortex. In the present study, we show that radial glial cells in the developing cerebral cortex selectively express a BLBPpromoter-EGFP, EYFP, or dsRed2 transgene. Analysis of several lines of BLBP-EGFP, EYFP, or dsRed2 transgenic mice indicates that expression of fluorescent protein is restricted to radial glia and astrocytes of the developing and mature brain, respectively. No neuronal expression of the transgene was detected.

BLBP expression molecularly defines radial glia in the developing brain. Radial glial cells emerge from the undifferentiated telencephalic neuroepithelium and can undergo several modes of differentiation. During very early stages of cortical development symmetric division of radial glia augments the pool of radial glial cells (Kriegstein and Gotz, 2003; Noctor et al., 2004). These cells span the entire width of the cortical wall with their thin, elongated processes. Radial glia then begin to divide asymmetrically to generate neurons and these newly generated neurons use radial glial processes as migrational guides to navigate from the ventricular zone to the cortical plate (Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001). The neurons generated by asymmetric division of radial glia loose the expression of radial glial markers such as BLBP or RC2. During late stages of cortical development, as neuronal generation and migration dwindles, radial glia transform into intermediate precursors or astrocytes (Schmechel and Rakic, 1979; Levitt et al., 1981; Hunter and Hatten, 1995; Noctor et al., 2004). In the mature cerebral cortex, some of the astroglial cells in the subgranular zone of the hippocampus and the subventricular zone of the lateral ventricles display similar functional characteristics as the embryonic radial glia and continue to function as neural stem cells (Builla and Lim, 2004). How these diverse aspects of radial glial differentiation are coordinated during the emergence of the cerebral cortex is not fully understood, partly as a result of the difficulties in clearly identifying radial glia in the developing brain. BLBP-EGFP, EYFP, or -dsRed2 transgenic mice or transgene expression in organotypic brain slices can be useful in several ways to overcome these difficulties. First, fluorescent-labeled radial glia containing cell cultures can be obtained from the transgenic lines for in vitro studies. Second, distinct aspects of radial glial development can be studied in vivo in live brain slices from these transgenic mice by using time-lapse confocal microscopy (Gongidi et al., 2004; Schmid et al., 2004). However, the abundance of fluorescent-labeled radial glia and variations in the level of fluorescent reporters in these transgenic lines can make studying the fate of individual radial cells in vivo difficult. BLBP-EGFP, EYFP, or dsRed2 electroporated organotypic embryonic cortical slices expressing high levels of fluorescent protein only in a fraction of the radial glia may be better suited for real-time, extended time-lapse studies aimed at defining the mechanisms underlying development and differentiation of radial glia. Furthermore, we have observed that fluorescent intensity dwindles in our transgenic lines after a few generations. This may occur as a result of promoter inactivation or the lack of other critical regions in the minimal BLBP promoter used in the present study (Feng and Heintz, 1995; Anthony et al., 2004, 2005). Though the mechanisms underlying promoter silencing effects are unclear, promoter silencing in transgenic animals has been found to correlate with methylation of cysteine-rich promoter regions and repression of the chromatin structure marked by histone hyperacetylation (Chevalier-Mariette et al., 2003; Brooks et al., 2004). Together, these studies suggest that the BLBP-EGFP, EYFP, or dsRed2 transgenic mouse lines expressing fluorescent proteins in a radial glia-specific manner or electroporation of these transgenes in embryonic brains can be useful tools to investigate the mechanisms underlying the differentiation of radial glia and astrocytes.

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