A BAC Transgenic Mouse Model to Analyze the Function of Astroglial SPARCL1 (SC1) in the Central Nervous System

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KEY WORDS
cerebral cortex; astroglia; SPARCL1; SC1; fatty acid binding protein 7; fabp7

ABSTRACT
Extracellular matrix associated Sparc-like 1 (SC1/SPARCL1) can influence the function of astroglial cells in the developing and mature central nervous system (CNS). To examine SC1's significance in the CNS, we generated a BAC transgenic mouse model in which Sc1 is expressed in radial glia and their astrocyte derivatives using the astroglial-specific Blbp (Brain-lipid binding protein; [Feng et al., (1994) Neuron 12:895–908]) regulatory elements. Characterization of these Blbp-Sc1 transgenic mice show elevated Sc1 transcript and protein in an astroglial selective pattern throughout the CNS. This model provides a novel in vivo system for evaluating the role of SC1 in brain development and function, in general, and for understanding SC1's significance in the fate and function of astroglial cells, in particular. ©2008 Wiley-Liss, Inc.

INTRODUCTION

SC1 is a member of the SPARC (Secreted Protein Acidic and Rich in Cysteine) family of extracellular matrix-associated proteins, which, in addition to SC1 and SPARC, includes testicans 1–3, tsc36, QR1, and SMOCs. This family of proteins is defined by the presence of a highly acidic domain I, a follistatin-like domain, and an extracellular calcium (EC) binding domain [reviewed in (Brekken and Sage, 2000; Framson and Sage, 2004; Yan and Sage, 1999)]. The follistatin-like domain, which shares homology to a repeated domain in follistatin, consists of a Kazal-type serine protease inhibitor region and an EGF domain. The EC binding domain contains a canonical pair of Ca2+ binding EF-hand motifs (Yan and Sage, 1999). The N-terminal domains of each family member exhibit the greatest divergence, and testican and tsc36/FRP both contain unique C-termini domains not present in the other members of SPARC family (Yan and Sage, 1999).

SPARC family members appear to function primarily as anti-adhesion signals. For example, SC1/hevin has been shown to inhibit attachment and spreading of endothelial cells on fibronectin in vitro (Girard and Springer, 1996). Similarly, SPARC has been shown to inhibit the formation of focal adhesion contacts by a tyrosine phosphorylation-dependant pathway, resulting in reduced adhesivity of endothelial cells in vitro (Motamed and Sage, 1998; Murphy-Ullrich et al., 1999). SPARC can also regulate cell adhesion by modulating the production and activity of matrix metalloproteinases involved in ECM remodeling (Gilles et al., 1998), by binding and sequestering Ca2+ through its EC domain, or through its association with ECM components such as vitronectin and collagen types I, III, IV, and V (Maurer et al., 1995; Sasaki et al., 1998). Additionally, SPARC can function to inhibit cell cycle progression and modulate growth factor activity (Brekken and Sage, 2000, 2001; Brekken et al., 2004).

Although the SPARC family of proteins are expressed in the developing and adult CNS, their functional significance in the brain remains, for the most part, unclear (Brekken et al., 2004). Recent studies have identified that the selective expression of SC1 in radial glial strands spanning the cortical plate (CP) enables it to influence the terminal phase of neuronal migration in the developing cerebral cortex (Gongidi et al., 2004). In the adult cerebral cortex, SC1 is enriched in postsynaptic terminals and in perisynaptic glial processes surrounding the synapse (Lively et al., 2007). Following injury, reactive astrocytes rapidly upregulate SC1 (McKinnon and Margolskee, 1996; Mendis et al., 1996a; Lively et al., 2007). Although these studies suggest potentially important roles for SC1 in the development, maturation, and function of the brain, further elucidation of SC1’s significance has been hampered by the lack of methods to selectively modulate SC1 expression and function in vivo in cells that mainly express it, i.e., astroglia.

Here, we describe the generation and characterization of a bacterial artificial chromosome (BAC) transgenic mouse line that expresses Sc1 under the transcriptional control of the Brain lipid-binding protein (Blbp or fatty acid binding protein 7, Fabp7) regulatory elements (Feng et al., 1994; Xu et al., 1996). This promoter region has...
been well characterized and used successfully to drive gene expression selectively in radial glia and their astrocytic progeny from embryonic day 13 onwards in the developing CNS (Anthony et al., 2004, 2005; Feng and Heintz, 1995). Utilization of the BAC transgenic approach allows for selective expression of SC1 in BLBP+ astroglial cells under more stable conditions than traditional transgenic approaches (Heintz, 2001). Widespread expression of SC1 was achieved in astroglial cells of the cerebral cortex, hippocampus, midbrain, and cerebellum in these mice. Generation of these mice provides a novel tool for the analysis of the role of SC1 in distinct compartments of the developing and adult mammalian brain.

METHODS
Construction of SC1-IREs-EYFPm BAC Transgene Construct

SC1-IREs-EYFP
m
pA was cloned into pCMV-Zeo (Invitrogen, V50120). CMV-Zeo was then replaced with floxed Neo cassette from p452 (a gift from Dr. N. Copeland of NCI) to introduce Neo as a selection marker for recombineering and to provide additional unique sites of restriction endonuclease to ligate with homologous arms for targeting. The SC1-IREs-EYFP
m
-pA was then inserted into Blbp exon 1 within C57 mouse BAC (RP23-81N21). With the deletion of 160 bp of exon 1 and part of intron 1, this transgene starts transcription after 39nt from the Blbp transcription initiation site (Fig. 1A).

Generation of BAC Transgenic Mice

Purified BAC was used for pronuclear microinjection of fertilized eggs from the CBA X C57Bl6 mouse strain at the UNC Animal Model core facility. Fertilized ovum was subsequently implanted into pseudo-pregnant females and offspring were analyzed for the insertion of was subsequently implanted into pseudo-pregnant at the UNC Animal Model core facility. Fertilized ovum of fertilized eggs from the CBA X C57Bl6 mouse strain under identical conditions. All procedures were carried out in accordance with NIH guidelines and The University of North Carolina’s Animal Care and Use Committee Guidelines.

In Situ Hybridization

SC1 probe spans sequence #902–1832 of the mouse SC1 cDNA sequence. The SC1 in situ construct was generated by RT-PCR from mouse brain cDNAs using SC1 specific primers (5’ GGC TGA AGA AAG CCA GAC AC 3’ and 5’ CTG CAG TTG TG 3’) and the 930 bp amplified product was cloned into pBluescript SK(+). After linearization with EcoRV, antisense transcripts were produced using T7 RNA polymerase. In situ hybridization on brain sections from Blbp-SC1 transgenic and wildtype control mice was performed as previously described (Anton et al., 2004) at the UNC Neuroscience Center’s in situ hybridization core facility. All images were captured under identical conditions using an Axios-Cam camera attached to a Zeiss Axioplan 2 microscope. The relative levels and spatial distribution of transgene expression was determined by reviewing multiple sections from different Blbp-SC1 transgenic lines and by comparison to sense control labeled slides.

Quantitative Real-Time-PCR

To assess the relative expression levels of SC1 in the Blbp-SC1 transgenic lines, RNA was extracted from Blbp-SC1 transgenic or wildtype cortex and cerebellum using Trizol (Invitrogen). High fidelity cDNA was generated from each RNA sample (SuperScript II, Invitrogen). QRT-PCR reaction samples were prepared as a mixture containing Sybergreen POWER Universal Master MixTM (Synthetegen), forward and reverse primers for SC1 or Gapdh, and equal amounts of cDNAs. The following primers were used: Gapdh, 5’-TGTGTCGTCGGATCTGGC-3’ and 5’-CCTGTTACCCATCTTGA-3’; SC1, 5’-GGCTGAAAGGCCAGACAC-3’ and 5’-GTGTCCTTCTTGTTGCCAT-3’. Reactions were performed using an Applied Biosystems Prism 9700 PCR machine. Relative expression of SC1 levels (SC1/Gapdh) in wildtype and Blbp-SC1 samples was determined as described by Pfaffl 2001.

Immunohistochemistry and Immunoblotting

Wildtype and Blbp-SC1 brains were harvested and processed for immunohistochemistry or immunoblotting as described earlier (Gongidi et al., 2004; Schmid et al., 2003). Briefly, brain tissue was either fixed in 4% paraformaldehyde for immunohistochemistry or flash frozen for Western blot analysis. Fixed brains were sagittally sectioned (30 µm thick) and processed for immunolabeling with a rat anti-SC1 antibody [11–59, generous gift of Dr. E. Helene Sage, The Hope Heart Institute]. Immunoreactivity was detected with Cy2 conjugated, anti-rat secondary antibodies (Jackson Immunoresearch). For Western blot analysis, harvested brains were homogenized in lysis buffer [20 mM Tris, pH 7.4, 150 mM NaCl, 10% Glycerol, 1% NP40, 0.25% Deoxycholate, 0.1% SDS, and protease inhibitors (Sigma)]. Equal amounts of proteins were then separated on 4–20% gradient SDS-PAGE gels and immunoblotted with anti-SC1 antibodies as described in Gongidi et al., 2004.
RESULTS

Generation of SC1-IRES-EYFP<sub>m</sub> BAC Transgenic Mouse Model

To test SC1 functions in the developing and mature brain, we have generated a BAC transgenic mouse model where SC1/SPARCL1 is expressed specifically in radial glial cells and their astrocyte derivatives using the astroglial specific Blbp (Brain lipid-binding protein or fatty acid binding protein 7, Faby<sub>7</sub>) regulatory elements. Disrupted normal SC1 expression in the developing and mature astroglial cells in these Blbp-Sc1 transgenic mice provides a framework to evaluate the role of astroglial SC1 in the brain. Previous studies have shown that the Blbp regulatory elements can drive gene expression in an astroglial-specific manner starting around embryonic day 13 (Anthony et al., 2004, 2005; Feng and Heintz, 1995). A SC1-IRES-EYFP<sub>m</sub>-pA construct containing Sc1, separated from the enhanced, membrane targeted yellow fluorescent protein (EYFP<sub>m</sub>) by an internal ribosomal entry signal (IRES), was generated and cloned into pCMV-Zeo (Invitrogen, V50120). CMV-Zeo was then replaced with floxed Neo cassette from p452 (a gift from Dr. N. Copeland of NCI) to introduce Neo as a selection marker for recombineering and to provide additional unique sites of restriction endonuclease to ligate with homologous arms for targeting. The Sc1-IRES- EYFP<sub>m</sub>-pA was then inserted into Blbp BAC clone (RP23-81N21). With the deletion of 160 bp of exon 1 and part of intron 1, the transgene starts after 39nt from the Blbp transcription initiation site (Fig. 1A). Prior to generation of BAC-transgenic...
mice, Blbp-Sc1 plasmids were electroporated into the ventricles of embryonic day 15 mice to ensure that transgene expression in the developing cerebral cortex was restricted to radial glial cells (Schmid et al., 2006). The purified BAC construct was used for pronuclear microinjection of fertilized eggs from C57B16 strain mice at UNC Animal Models core facility. Six founder lines were identified by Southern blot analysis of tail DNA using a probe for the Blbp-Sc1 transgene (Fig. 1B).

Independent breeding lines were established from four of these lines (named Blbp-Sc11–4). Expression of this transgene does not appear to affect the overall viability of transgene positive progeny.

**Characterization of Blbp-Sc1 Transgene Expression**

We initially performed in situ hybridization to examine the Sc1 expression pattern in the CNS of Blbp-Sc1 transgenic lines and control wildtype mice. Compared to wildtype cerebral cortex, elevated Sc1 expression is evident throughout the cortex (Fig. 2B and C). Although there was a marked elevation in expression in all regions of the cerebral cortex, the most striking rise in Sc1 expression was seen in the region of occipital and parietal cortex. In the hippocampus, widespread increase in astroglial Sc1 expression was noticed, especially in the regions of the subiculum, CA3, and dentate gyrus (Fig. 2D and E). In the striatum, stellate shaped, multipolar astroglial cells prominently expressed Sc1 (Fig. 2F and G). Furthermore, significant upregulation of Sc1 in Bergmann glia of the cerebellum was also evident in these transgenic lines (Fig. 2H–K). Labeling with Sc1 sense control probes showed no detectable signal (data not shown).

To further demonstrate that introduction of the Blbp-Sc1 transgene resulted in an upregulation of Sc1 expression within the CNS of these transgenic lines, we performed quantitative RT-PCR (QRT-PCR) analysis of Sc1 expression in the cortex and cerebellum of Blbp-Sc1 transgenic mice compared to wildtype controls (Fig. 2L). Data shown are mean ± SD. Asterisks indicates significance compared with controls at P < 0.001 (Student's t test). S, subiculum; DG, dentate gyrus; ML, molecular layer; PL, Purkinje layer; GL, granule layer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

![Fig. 2. Patterns of Sc1 expression in Blbp-Sc1 transgenic mice. In situ hybridization analysis demonstrated elevated expression of Sc1 transcript in distinct regions of the CNS of Blbp-Sc1 transgenic mice. Sc1 expression in Blbp-Sc1 transgenic mice (C, E, G, I, K) was compared with age-matched controls (B, D, F, H, J). Regions examined are indicated on a schematic of the sagittal view of adult brain (A) Sc1 expression is upregulated in cerebral cortex (B, C); hippocampus (D, E); striatum (F, G); cerebellum (H–K). Arrowheads (G) indicate Sc1 overexpressing multipolar astrocytes in the striatum. Higher magnification images of the cerebellum indicates prominent Sc1 overexpression in Bergmann glia with elongated processes in Blbp-SC1 mice (arrowheads, K). Quantitative RT-PCR analysis with Sc1 specific primers reveals a significant increase in the level of Sc1 transcript in both the cerebral cortex and cerebellum of Blbp-Sc1 transgenic mice compared to wildtype controls (L). Data shown are mean ± SD. Asterisks indicates significance compared with controls at P < 0.001 (Student's t test). S, subiculum; DG, dentate gyrus; ML, molecular layer; PL, Purkinje layer; GL, granule layer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]](#)
increase in SC1 protein expression, we performed immuno blot analysis of whole cell extracts from Blbp-Sc1 transgenic mice sections of adult cortex and cerebellum were immunolabeled with anti-SC1 antibodies. Increased levels of SC1 are noticed in astroglial cells of Blbp-Sc1 transgenic mice within both the cortex (B) and the cerebellum (D), as compared to basal levels of SC1 expression in wildtype mice (A [cortex], C [cerebellum]). Arrowheads (D) indicate Bergmann glia of the cerebellum. Immunoblot analysis of Blbp-Sc1 brains demonstrates an increase in the levels of the 116 kDa SC1 protein (E). Arrowhead (E) indicates the unprocessed precursor of SC1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Fig. 3. Elevated expression of SC1 in the cerebral cortex and cerebellum of Blbp-Sc1 transgenic mice sections of adult cortex and cerebellum were immunolabeled with anti-SC1 antibodies. Increased levels of SC1 are seen in astroglial cells of Blbp-Sc1 transgenic mice within both the cortex (B) and the cerebellum (D), as compared to basal levels of SC1 expression in wildtype mice (A [cortex], C [cerebellum]). Arrowheads (D) indicate Bergmann glia of the cerebellum. Immunoblot analysis of Blbp-Sc1 brains demonstrates an increase in the levels of the 116 kDa SC1 protein (E). Arrowhead (E) indicates the unprocessed precursor of SC1. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

Generation and characterization of a BAC transgenic mouse model in which astroglial selective over-expression of SC1 is achieved will facilitate the analysis of SC1 functions, particularly those mediated through astroglia, in the development and maturation of distinct brain structures. Considering the known functional redundancy of SPARC family of proteins in the brain and the entailing difficulties in using traditional knock-out approach to delineate functional significance of SPARC family members (Gilmour et al., 1998; McKinnon et al., 2000; Roll et al., 2006), this approach provides a useful model to analyze SC1 function in a cell type specific manner in the brain.

SC1’s expression in the developing, mature, and injured brain suggests that it may influence multiple cellular processes during distinct stages of brain development and function. In the embryonic brain, SC1 is prominently expressed in radial glia where it functions to terminate radial glial guided neuronal migration by reducing the adhesivity of neurons to the radial glial scaffold (Gongidi et al., 2004). Furthermore, SC1 is also expressed in the proliferative ventricular zone (VZ) of the embryonic cortex where it may influence the dynamics of radial glial cell proliferation and survival (Girard and Springer, 1995, 1996; Gongidi et al., 2004). Additionally, the presence of anti-adhesive signals such as SC1 in the VZ may play a role in turning VZ
directed subpopulation of neurons back on a trajectory towards the CP (Nadarajah et al., 2002; Yokota et al., 2007). The selective SC1 expression in the VZ and CP, and the differential function of SC1 in distinct cerebral wall domains could, in turn, provide a mechanism for the coordination of neural precursor proliferation and neuronal placement in the developing neocortex. SC1 is also expressed in Bergmann glia of the developing cerebellum, where selective transport of SC1 may influence glial guided migration or modulation of synaptic activity (Brekken et al., 2004; Mothe and Brown, 2000). In the mature brain, SC1 expression persists in radial glial derivatives, that is, astrocytes. SC1 expression in astrocytes adjacent to synapses is hypothesized to modulate synaptic function (Lively et al., 2007). These varied functions of SC1 that are mediated through radial glia or astrocytes could be further evaluated using Blbp-Sc1 transgenic lines where normal expression of SC1 is selectively disrupted in these cells.

Following brain injury, SC1 is rapidly upregulated in reactive astrocytes around the site of injury (Lively and Brown, 2007; McKinnon and Margolskee, 1996; Mendis et al., 1996a,b). This upregulation in astrocytes may enhance the repair process following injury and the Blbp-Sc1 transgenic mouse model could help to evaluate this possibility. Although the exact levels of SC1 upregulation necessary to induce repair mechanism follow focal trauma has not been established, the Blbp-Sc1 transgenic mouse model induces an over 50-fold induction of Sc1 mRNA expression in the adult cerebral cortex and over 5-fold induction in the cerebellum. Furthermore, SC1 may also be involved in the pathogenesis of multiple neural traumas. For example, proteomic analysis of cerebrospinal fluid from multiple sclerosis patients showed an upregulation of SC1 expression (Scalabrin et al., 2007). SC1 may also function as a tumor suppressor and is aberrantly expressed in brain tumors (Framson and Sage, 2004; Sullivan and Sage, 2004). SC1’s ability to remodel extracellular matrix, regulate cell cycle progression, and act as substrate for apoptosis regulators, such as caspase-3, may be highly relevant to its function in these aberrantly proliferating and invasive brain tumor cells (Hambrock et al., 2003; Ju et al., 2007; Sullivan et al., 2006). Blbp-Sc1 transgenic lines could be crossed with various mouse brain tumor models (Uziel et al., 2005; Xiao et al., 2002; Zurawel et al., 2000) to evaluate if enhanced astroglial SC1 expression could modulate the formation and progression of gliomas.

In summary, SC1 expression in the astroglial cells of the developing, mature, and injured brain is highly suggestive of dynamic functions of this protein in the CNS. The generation of a Blbp-Sc1 transgenic mouse model described here provides a useful system for determining in vivo how astroglial expression of SC1 contributes to the construction and function of distinct brain regions.

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GLIA