Floxed Allele for Conditional Inactivation of the Voltage-Gated Sodium Channel β1 Subunit Scn1b

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Summary: The voltage-gated sodium channel gene Scn1b encodes the auxiliary subunit β1, which is widely distributed in neurons and glia of the central and peripheral nervous systems, cardiac myocytes, skeletal muscle myocytes, and neuroendocrine cells. We showed previously that the Scn1b null mutation results in a complex and severe phenotype that includes retarded growth, seizures, ataxia, and death by postnatal day 21. We generated a floxed allele of Scn1b by inserting loxP sites surrounding the second coding exon. Ubiquitous deletion of the floxed exon by Cre recombinase using CMV-Cre-transgenic mice produced the Scn1bdel nulls and confirms the in vivo inactivation of Scn1b. Conditional inactivation of the floxed allele will make it possible to circumvent the lethality that results from complete loss of this gene, such that the physiological role of Scn1b in specific cell types and/or specific developmental time points can be investigated.

Key words: Scn1b; β1; conditional allele; Cre/loxP

Voltage-gated sodium channels initiate and propagate action potentials in excitable cells and are key modulators of electrical signaling (Catterall, 2000). Sodium channels are composed of a central, pore-forming α subunit and two auxiliary β subunits. The β subunits do not form the pore but play important roles in channel gating, channel cell surface expression, and cell-to-cell communication (Isom, 2002; Meadows and Isom, 2005). Sodium channels are unique among voltage- and ligand-gated ion channels in that their auxiliary β subunits both modulate channel function and serve as cell adhesion molecules (CAMs) through their extracellular immunoglobulin (Ig)-like domains. We and others have demonstrated that β subunits, especially β1 (Scn1b), function as CAMs: Scn1b interacts with the extracellular matrix protein tenascin-R to influence cell migration (Xiao et al., 1999); Scn1b participates in homophilic cell adhesion, resulting in cellular aggregation and ankyrin recruitment (Malhotra et al., 2000, 2002); Scn1b interacts heterophilically with the CAMs contactin, neurofascin-155, neurofascin-186, and NrCAM (Kazarinova-Noyes et al., 2001; McEwen and Isom, 2004; McEwen et al., 2004; Ratcliffe et al., 2001); Scn1b interacts with receptor phosphotyrosine phosphatase β (Ratcliffe et al., 2000); Scn1b interacts with contactin and neurofascin-186 result in increased channel cell surface expression (Kazarinova-Noyes et al., 2001; McEwen and Isom, 2004; McEwen et al., 2004); and Scn1b promotes neurite extension as a result of homophilic adhesion (Davis et al., 2004). Mutations in the extracellular Ig domain of Scn1b are linked to generalized epilepsy with febrile seizures plus (GEFS+) and/or temporal lobe epilepsy in human patients (Audenaert et al., 2003; Scheffer et al., 2006; Wallace et al., 1998, 2002). Thus, we propose that, as a channel modulator, Scn1b plays important roles in the control of electrical signaling and, as a CAM, Scn1b acts as a critical communication link between extra- and intracellular signaling molecules.

β1 is encoded by a single gene in the mammalian genome, SCN1B (Makita et al., 1994a), that is expressed in central and peripheral neurons (Altman, 1972a,b; Sashihara et al., 1995; Singh, 1977; Sutkowski and Catterall, 1990), including nodes of Ranvier in myelinated axons (Chen et al., 2004), glia (Blackburn-Munro and Fleetwood-Walker, 1999; Oh and Waxman, 1994, 1995; Oh et al., 1995, 1997), cardiac and skeletal muscle myocytes (Maier et al., 2004; Makita et al., 1994a; Malhotra et al., 2001), and neuroendocrine cells (Kazen-Gillespie et al., 2000). These data suggested that complete loss of

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SCN1B in vivo would produce a complex and severe phenotype. In support of this, we showed that Scn1b null mice are ataxic, display spontaneous generalized seizures, are growth retarded, and die by 3 weeks of age (Chen et al., 2004). To understand the physiological role of Scn1b in normal development as well as in brain and heart disease, and to circumvent the lethality associated with complete loss of this gene, we generated mice carrying the conditional Scn1b\textsuperscript{floxed} allele. Scn1b is composed of five coding exons spanning \~10 kb of genomic DNA (Makita et al., 1994b). We introduced loxP sites at positions 190 bp upstream and 220 bp downstream, respectively, of exon 2 in Scn1b cloned from a mouse 129 library, as described previously (Chen et al., 2004; Fig. 1). Exon 2 was chosen over exon 3 for targeting [as in our previous targeting experiment (Chen et al., 2004)] so as to leave intron 3 intact for expression of the splice variant β1A, retaining this.

FIG. 1. Targeting of Scn1b. (a) Schematic of gene-targeting strategy: The targeting construct contained exons 1, 2, and 3 (E1–E3), with E2 flanked by loxP sites (arrows) and an inverted PGK-neocassette flanked by FRT sites (pentagons). There was 2.2 kb of homologous sequence in the left (5') arm and 4.1 kb in the right (3') arm. The 5' and 3' Southern probes, as well as diagnostic SpeI (S) restriction sites are indicated. (b) Southern blots of ES cell genomic DNA digested with SpeI. Lanes 1 and 2: blot probed with 5' probe shown in panel a. Lane 1, correctly targeted. Lane 2, wild type. Lanes 3 and 4: blot probed with 3' probe shown in panel a. Lane 3, correctly targeted. Lane 4, wild type. (c) PCR of ES cell genomic DNA showing correct targeting of the left (5') loxP site. Lower band (310 bp), endogenous allele. Upper band (430 bp), correctly targeted allele.
intron, in the targeted mice prior to Cre recombinase expression (Kazen-Gillespie et al., 2000). Exon 2 of Scn1b contains the amino acid sequence: GAVLVSAAWGCV EVDSETAVYGMFTFKICSLCKRSSSETATFPETWTFROKG TEEFK, that includes the amino-terminus of the mature Scn1b protein after removal of the signal sequence (G) as well as the first cysteine residue of the Ig loop domain (C). Thus, following deletion of this exon, we predicted that it would be impossible for functional Scn1b protein to be expressed. The floxed exon and two homology arms of 2.2 and 4.1 kb each, containing exons 1 and 3, respectively, were assembled in a vector containing a neocassette flanked by FRT sites (Fig. 1a). The FRT sites were included to permit in vivo deletion of the neocassette if its presence was found to interfere with the expression of the targeted allele (Dymecki, 1996).

The targeting vector was electroporated into PAT-5 embryonic stem (ES) cells (Chen et al., 2004; Domino et al., 2001) and 480 neomycin-resistant colonies were recovered. These colonies were screened by Southern blot using both a 5′ and 3′ probe, located within the 5′ homology arm, and a 3′ probe, located downstream of the 3′ homology arm, as indicated in Figure 1a. Eleven of the 480 colonies screened were correctly targeted as defined by Southern blot analysis. Figure 1b shows Southern blots of a correctly targeted ES cell clone. We found that the 5′ loxP site was lost in some clones during targeting and thus screened for retention of this site by PCR in all of the clones determined to be correctly targeted by Southern blot (Fig. 1c). Using these criteria, nine correctly targeted recombinants were obtained, giving a final targeting efficiency of ~2%. These ES cell clones were subjected to chromosome analysis, and ultimately two (1F1 and 5A2) were injected into C57BL/6 blastocysts to produce chimeric mice. Germline transmission was obtained from the 1F1 line but not from the 5A2 line.

Crossing chimeric mice to strain C57BL/6 mice generated Scn1bneo/+ mice that were heterozygous for the neoallele shown in Figure 1a. To determine whether the neocassette interfered with Scn1b expression, these mice were then bred to homozygosity. In contrast to other conditional null sodium channel gene targeting experiments (Levin and Meisler, 2004), Scn1bflpneo mice exhibited a normal phenotype, lived normal life spans, and bred normally. These results suggested that neither the neocassette nor the loxP sites present in the targeted allele affected Scn1b expression. Thus, we elected to proceed directly to the Cre recombinase breeding step (Fig. 1a) and leave the neocassette intact in the targeted allele.

To determine whether deletion of exon 2 would inactive Scn1b, homozygous Scn1bflpflp mice were crossed with CMV-Cre-transgenic mice. The predicted deleted allele, shown in Figure 2, was detected in heterozygous offspring (Scn1bflpflp/+ mice) by amplification of a 436-bp fragment from genomic DNA using the primers loxP-5′ and BamH1-fwd (Fig. 2a). Intercrosses between heterozygous Scn1bflpflp/+ mice generated homozygous mice (Scn1bflpflp/flp) that exhibited severe seizures beginning at approximately postnatal day 10, retarded growth (Fig. 2b), ataxia, and 100% lethality by 3 weeks of age, a phenotype that was indistinguishable from mice carrying the Scn1b null allele (Chen et al., 2004). All of the affected mice were homozygous for the deleted allele (Fig. 2a, del/del).

We performed in situ hybridization analysis to determine the extent of Scn1b inactivation in Scn1bflpflp brains at P17-18 (Fig. 2c). Scn1b mRNA was extensively reduced in all brain areas compared to Scn1bflpflp/+ mice. Western blot analysis showed that levels of Scn1b protein expression in brain membrane preparations from Scn1bflpflp/+ mice were similar to wild-type mice (Fig. 2d). Importantly, Scn1bflpflp/+ mice showed no Scn1b protein expression, similar to Scn1b null mice (Fig. 2d; Chen et al., 2004), and exhibited an indistinguishable behavioral phenotype. Thus, the behavioral and molecular phenotypes of Scn1bflpflp/+ mice demonstrate successful Cre-mediated inactivation of Scn1b.

In conclusion, we generated a floxed allele of Scn1b that can be inactivated by expression of Cre recombinase. The floxed allele produces normal levels of Scn1b protein and homoygotes, for this allele does not exhibit behavioral or molecular abnormalities. In vivo deletion of exon 2 by Cre recombinase results in complete loss of Scn1b expression. Mice carrying the Scn1bflpflp allele will be critical to the future dissection of the physiological consequences of Scn1b inactivation in specific cell types and/or at specific developmental time points, and thus to the understanding of the mechanism of Scn1b function.

MATERIALS AND METHODS

Materials

PAT-5 ES cells were obtained from the University of Michigan Transgenic Animal Model Core Laboratory (Domino et al., 2001). CMV-Cre-transgenic mice were obtained from Dr. Anand Swaroop at the University of Michigan. The plasmid, ploxPFlpneo (Hiraoka et al., 2006), containing two loxP sites and a neocassette flanked by FRT sites, was obtained from the University of Michigan Transgenic Animal Model Core Laboratory. A rabbit polyclonal antibody raised against the C-terminal of Scn1b (Oyama et al., 2006) was obtained from Dr. N. Nukina, Dr. Oyama, and Dr. Miyazaki at the RIKEN Brain Institute.

Construction of the Targeting Vector and Screening of ES Cells

Scn1b was cloned from a mouse genomic 129 library (Stratagene, LaJolla, CA) using the full-length rat Scn1b cDNA as a probe, as previously described (Chen et al., 2004). A 2.2-kb genomic fragment containing a 5′ flanking region and exon 1 was inserted into the Kpn1 site of ploxPFlpneo (Hiraoka et al., 2006). A 540-bp fragment containing exon 2 was inserted into the BamH1 site of ploxPFlpneo. Finally, a 4.1-kb fragment containing exon...
FIG. 2. Mouse characterization. (a) Genotyping—Left panel: I. PCR of tail DNA using primer pair loxP-5' and BamH1-fwd. The deleted allele (del) generated a 436-bp product and the floxed allele (flox) a 1,049 bp product, as indicated by the arrows. The endogenous allele (+) is not detected with this primer pair. II. PCR of genomic DNA using primer pair loxP-5' and 201-3'. The endogenous allele (+) generated a 320-bp product, and the floxed allele (flox) generated a 540-bp product. The deleted allele (del) is not detected with this primer pair. III. PCR of tail DNA using primer pair CreF and CreR to detect Cre recombinase. The Cre allele generated a 850-bp band. Right panel: locations of primers and expected PCR bands from endogenous, floxed, and deleted alleles. (b) Body weight: I: comparison of typical Scn1b^{flox/+} and Scn1b^{del/del} littermate mice at P17. II: body weights of P16-17 Scn1b^{flox/+} vs. Scn1b^{del/del} littermate mice. Mean and SEM are presented. For Scn1b^{flox/+}: mean = 7.95 ± 0.07 g; for Scn1b^{del/del}: mean = 3.93 ± 0.19 g, P < 0.0001. (c) In situ hybridization: coronal brain slices from P17 Scn1b^{flox/+} and Scn1b^{del/del} littermate mice were prepared for in situ hybridization with 35S-labeled Scn1b sense and antisense mRNA probes as described in Material and Methods. I. Scn1b^{flox/+} brain probed with 35S-labeled Scn1b antisense probe. II. Scn1b^{del/del} brain probed with 35S-labeled Scn1b antisense probe. 35S-labeled Scn1b sense probes gave no signal above background on brain slices from Scn1b^{flox/+} brains, similar to the image shown in II (data not shown). (d) Western blot: Brain membranes were prepared from P17-18 Scn1b^{flox/+}, Scn1b^{del/del}, Scn1b wild-type, and Scn1b null mice. Western blot analysis was performed on equal aliquots of protein as described in Materials and Methods. Rat brain membranes, that had been previously prepared and stored at -80°C, were used as a positive control. An anti-Scn1b rabbit polyclonal antibody (1:500 dilution, obtained from Dr. N. Nukina (Oyama et al., 2006)) was used to detect Scn1b protein. Arrow indicates migration of wild-type Scn1b subunits. Molecular weights are indicated in kilodaltons.

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3' and the 3' flanking region was inserted into the Swa1 site of ploxPFlpneo. These manipulations resulted in insertion of the FRT-neo-FRT cassette into intron 2 of Scn1b in the 3' to 5' orientation relative to the Scn1b coding sequence as well as the insertion of loxP sites in the introns flanking exon 2. Probes to detect homologous recombination on the 5' and 3' arms following digestion of genomic DNA with SpeI were the same as those used in our previous Scn1b targeting experiment (Chen et al., 2004). PAT-5 ES cells were electroporated and 480 clones were screened by Southern blot using 5' and 3' probes as previously described (Chen et al., 2004). We found that in many clones the 5' loxP site was lost during targeting. Thus, we also developed a PCR experiment using primers flanking this loxP site (loxP-5': GGTACTCACCAGTGACATCCTC, and 201-3': AGGCTGAGGATCTGCGGACAGTGACG) to determine correct targeting. Nine clones were identified as correctly targeted, subjected to chromosome analysis, and two clones (1F1 and 5A2) injected into C57BL/6 blastocysts. High-percentage chimeric animals were obtained from the 1F1 breeding and bred to C57BL/6 mice to produce heterozygous animals. Genotyping was performed by PCR using tail DNA, as described later. ES cell electroporation and colony selection, injection of blastocysts, and generation of chimeric mice were provided as a service of the Transgenic Animal Model Core Laboratory at the University of Michigan. All experiments were carried out in accordance with the guidelines for Animal Care of the University of Michigan.

PCR Analysis

Floxed and deleted mice were genotyped using two sets of primers: a set of primers external to the loxP sites that amplified a 1,049-bp band corresponding to the floxed allele or a 436-bp band corresponding to the deleted allele (Fig. 2a, right panel; loxP-5': described earlier and BamH1-fwd: 5'-CCGCTGAGGCTCTATCGGCTCTGGAGG-3'); and a set of primers which amplified a region surrounding the 5' loxP site (Fig. 2a, right panel; loxP-5': and 201-3', described earlier). This second set of primers amplified a 540-bp band from the floxed allele, a 320-bp band from the endogenous allele, and no band from the deleted allele. Cre recombinase was detected by PCR using the primers CreF-2: 5'-TCCAATTTACTGACCGTACACC-3' and CreR: 5'-GGTTATCTCTGACCAGGACGTACATC-3', which amplified a 850-bp band (Fig. 1c).

In Situ Hybridization Analysis

Generation of mRNA probes. A cDNA template for generation of Scn1b sense and antisense probes was prepared by RT-PCR using mouse brain total RNA as template and the primers, Scn1b-probe-Xho5' (5'-CAACTGGGCGGGGAGGCGGCTCGTGGG-3') and Scn1b-probe-Kpn3' (5'-GAAGTGACGAGCCGCCCATTG-3'), based on the mouse Scn1b cDNA sequence NM 011322. The resulting PCR product was subcloned into pBluescript and the correct DNA sequence was confirmed by sequencing. To generate sense probe, the template was linearized with Kpnl, and T3 RNA polymerase (Promega, Madison, WI) was used in the labeling reaction. To generate antisense probe, the template was linearized with XbaI, and T7 RNA polymerase (Promega) was used in the labeling reaction. 35S-labeled mRNA probes were prepared in the following mixture, incubated for 2 h at 37°C (total volume: 25 μl): 5 μl H2O, 4 μl 35S-UTP (SJ603, 20 mCi/ml, Amersham, Piscataway, NJ), 2 μl 35S-CTP (SJ40382, 40 mCi/ml, Amersham), 5 μl 5' transcription buffer, 2.5 μl 100 mM dithiothreitol (DTT), 2 μl linearized cDNA (0.5–1 μg), 1 μl 10 mM GTP, 1 μl 10 mM ATP 1 μl RNase inhibitor (>20 U), 1.5 μl RNA polymerase, as indicated earlier. One microliter of RNase-free DNase was then added for 15 min at room temperature. Labeled probes were separated from free isotope by passing over a Micro Bio-spin column (Bio-Rad No. 732-6200, Hercules, CA) according to the manufacturer's instructions. DTT was added to the eluted probe to a final concentration of 10 mM. The specific activity of each probe was determined by scintillation counting. Aliquots of each labeled probe were stored at −80°C. For use, probes were diluted in hybridization buffer, such that 1 × 10^6 - 2 × 10^6 dpm were applied to each brain slice.

Hybridization and detection. Scn1bfloxed/+ and Scn1bdel/del littermate mice were killed at P17-18. Fresh frozen mouse brains were sectioned at 20 μm on a cryostat and mounted on Fisher Superfrost plus slides. Slides were then stored at −80°C. For in situ hybridization, slides were removed from the −80°C freezer, placed directly into 4% paraformaldehyde for 1 h at room temperature, and then washed three times in 2× SSC for 5 min each. Slides were then incubated in 0.1 M triethanolamine, pH 8.0, containing 0.25% vol/vol acetic anhydride, for 10 min at room temperature. Slides were washed briefly in water and dehydrated through a graded series of ethanol (50–100%) and allowed to air dry. Each brain slice on the slide was hybridized with 70 μl of 35S-labeled sense or antisense cRNA probe (containing 1 × 10^6 - 2 × 10^6 dpm) in 50% formamide hybridization buffer, covered, and incubated overnight in a Saran-wrapped box in a 55°C hybridization oven. The next day, coverslips were removed and slices were washed three times in 2× SSC followed by RNase A digestion (200 μg/ml) for 1 h at 37°C. Slides were then washed in 2× SSC, 1× SSC, and 0.5× SSC, incubated in the 0.1× SSC for 1 h at 70°C, rinsed with water, dehydrated through a graded series of ethanol (50–100%), and allowed to air dry. Labeled slides were exposed to Kodak film for 2–7 days at room temperature. Selected slides were coated with photographic emulsion (NTB2 Eastman Kodak, Rochester, NY) for 7–14 days at 4°C, developed, and counterstained with cresyl violet.

Western Blot Analysis

Scn1bfloxed/+ and Scn1bdel/del littermates, as well as Scn1b wild-type and null littermate mice (Chen et al., genesis DOI 10.1002/dvg

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