Biochemical and Biophysical Research Communications 410 (2011) 289–294

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Generation of a *Slc39a8* hypomorph mouse: Markedly decreased ZIP8 $Zn^{2+}/(HCO_3^-)_2$ transporter expression

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ARTICLE INFO

Article history:
Received 24 May 2011
Available online 31 May 2011

Keywords:
Zinc transporter
Bicarbonate transporter
Solute-carrier (SLC) family
Slc39a8 gene
ZIP8 transporter
Hypomorph mouse
Neo-mediated down-regulation of gene expression
Cadmium
In utero
Cre recombinase

ABSTRACT

Previously this laboratory has identified the mouse *Slc39a8* gene encoding the ZIP8 transporter, important in cadmium uptake. ZIP8 functions endogenously as a electroneutral $Zn^{2+}/(HCO_3^-)_2$ symporter, moving both ions into the cell. The overall physiological importance of ZIP8 remains unclear. Herein we describe generation of a mouse line carrying the *Slc39a8(neo)* allele, containing the *Frt*-flanked neomycin-resistance (*neo*) mini-cassette in intron 3 and *loxP* sites in introns 3 and 6. Cre recombinase functions correctly in *Escherichia coli* and in adeno-Cre-infected mouse fetal fibroblasts, but does not function in the intact mouse for reasons not clear. *Slc39a8(neo)* is a hypomorphic allele, because *Slc39a8(neo/neo)* homozygotes exhibit dramatically decreased ZIP8 expression in embryo, fetus, and visceral yolk sac – in comparison to their littermate wild-type controls. This ZIP8 hypomorph will be instrumental in studying developmental and in utero physiological functions of the ZIP8 transporter.

1. Introduction

Cadmium (Cd, Cd^{2+}) is classified by IARC as a "Category I" human lung carcinogen. Individuals at highest risk for Cd-induced lung cancer and chronic renal disease include cigarette smokers, those on a diet rich in high-fiber foods or contaminated shellfish, women having low body-iron stores, and malnourished populations [1–4]. It has long been known that Cd causes damage to the central nervous system, lung, bone, gastrointestinal tract, liver, ovary, testis, placenta, and developing embryo [5,6]. Chronic Cd exposures [7] cause renal proximal tubular metabolic acidosis and osteomalacia (renal Fanconi syndrome).

Recent studies in the mouse showed a relationship between a specific genotype (*Slc39a8* allelic differences) and phenotype (susceptibility to Cd-induced testicular necrosis). Starting with genetically "sensitive" vs "resistant" mice [8], our lab confirmed that the major locus (*Cdm*) was responsible for this trait [9]. We identified by positional cloning the *Slc39a8* gene, which encodes the metal transporter ZIP8, as the most likely candidate for the *Cdm* locus; high ZIP8 mRNA expression occurs in endothelial cells of the testicular vasculature in two Cd-sensitive mouse lines, whereas ZIP8 expression is negligible in this cell type from two Cd-resistant mouse lines [10]. We proved that the *Slc39a8* gene is indeed the *Cdm* locus by creating a bacterial artificial chromosome (BAC)-transgenic mouse line, *BTZIP8-3*. A 168.7-kb BAC, containing only the *Slc39a8* gene from a 129S6/SvEvTac (cadmium-sensitive) BAC library, was inserted into the Cd-resistant C57BL/6J genome; Cd treatment caused testicular necrosis in BAC-transgenic *BTZIP8-3* mice but not in non-transgenic littermates [11].

ZIP8 functions endogenously as an electroneutral $Zn^{2+}/(HCO_3^-)_2$ as well as a $Mn^{2+}/(HCO_3^-)_2$ symporter moving both ions into the cell [7,12,13]. Cd has a binding affinity for ZIP8 in the same range as zinc (Zn) [13]; thus, Cd is readily able to displace Zn and enter cells that express the functional ZIP8 transporter.

The next step in understanding ZIP8's endogenous functions was to generate a knockout mouse line. In building the *Slc39a8* knockout construct, we inserted the *Frt*-flanked neomycin-resistance gene (*neo*) into intron 3, along with *loxP* sites in introns 3 and 6. A hypomorphic phenotype was generated—in mice retaining the *neo* gene.

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doi:10.1016/j.bbrc.2011.05.134

Curiously, Cre recombinase-mediated *loxP* excision occurred in bacteria but not in the intact mouse; herein we examine possible reasons for this excision failure.

2. Materials and methods

2.1. Creation of the Slc39a8 construct

This lab previously had characterized a 168.7-kb BAC, in the middle of which is located *Slc39a8* as the only protein-coding gene [11]; the longest transcript of the mouse *Slc39a8* gene spans 64.7 kb [11,14]. From this BAC, we isolated a 9.7-kb subclone into pBluescript II SK(−) vector with a BAC subcloning kit (Gene Bridges; Dresden, Germany); this subclone contains *Slc39a8* exons 4–5–6 and portions of introns 3 and 6 (Fig. 1A, *top*). Following the protocol of the conditional-gene-knockout kit (Gene Bridges), we first inserted the *loxP-neo-loxP* template into the distal site in intron 6 by ET-recombineering [15]. Cre excision in *Escherichia coli* resulted in a single *loxP* site in intron 6, located 316 bp downstream of exon 6. Next, we inserted the *loxP-Frt-neo-Frt* template (Gene Bridges) into intron 3 by ET-recombineering (Fig. 1A, 2*nd line*); this template contains a phosphoglycerate kinase (PGK) promoter-derived *neo* mini-cassette flanked by two *Frt* sites plus one proximal *loxP* site [16]. The *neo* mini is needed for embryonic-stem (ES)-cell-positive selection for gene targeting. However, the *neo* cassette in many cases dampens gene expression; thus, it is common to include flanking *Frt* sites; FLP recombinase (expressed in FLP-transgenic mice) is then able to recombine with the two *Frt* sites and remove the *neo* mini-gene, as needed. If the *neo* gene is removed, this *loxP* site would be located 301 bp upstream of exon 4 (Fig. 1A, *3rd line*). This "floxed" allele (containing two *loxP* sites) in most cases is regarded as equivalent to the wild-type allele, and should have a minimal interfering effect on the targeted gene.

ES cells (129S6/SvEvTac) were electroporated with the linearized knockout construct, using standard methods. Neomycin-resistance clones were picked, expanded, and probed via Southern blot to screen for the correctly targeted clones (Fig. 1B). The genomic DNA was digested with *Sca*I; the Southern blot probe is described in Supplementary Data online.

The correctly targeted ES cell clone was microinjected into mouse blastocysts – with help from the University Cincinnati Gene Targeting Core Facility. Chimeric mice were generated and bred further with B6 mice. By genotyping agouti pups, germ-line transmission of the *Slc39a8(neo)* allele (Fig. 1A, 2*nd line*) was confirmed. Genotypes of the wild-type and *Slc39a8(neo)* alleles were verified by PCR. All genotyping primers are listed in Supplementary Data Table S1 online.

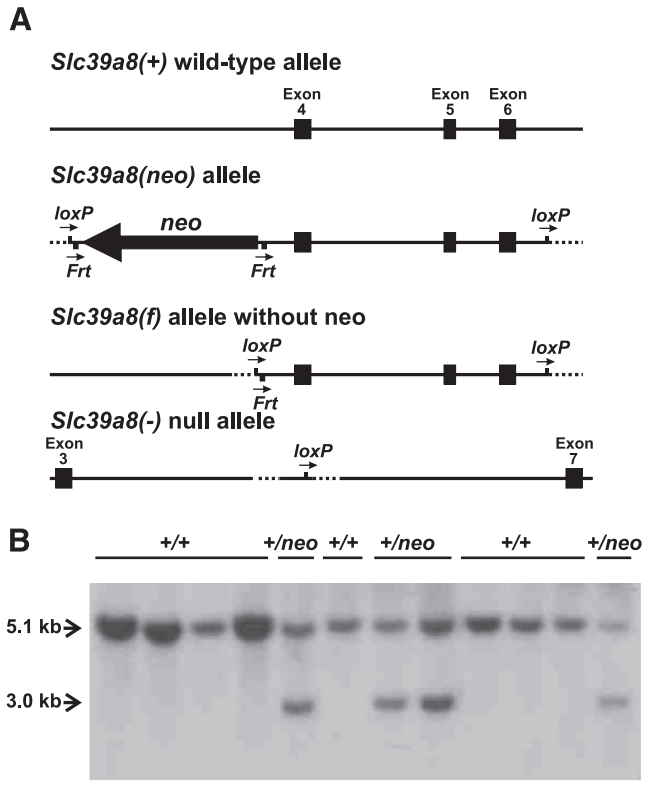

Fig. 1. Characterization of the *Slc39a8* alleles. (A) Diagram of the four *Slc39a8* alleles discussed. The mouse *Slc39a8* transcript (nine exons, eight introns) spans ~64.7 kb; total lengths of introns 3 and 6 are 6725 and 25,955 bp, respectively [11,14]. *Top*, *Slc39a8(+)* wild-type allele, showing exons 4 (170 bp), 5 (123 bp) and 6 (171 bp), introns 4 (1409 bp) and 5 (465 bp), and portions of introns 3 and 6. *Second line*, *Slc39a8(neo)* allele – proximal *loxP* site was inserted into intron 3, 301 bp upstream from start of exon 4, using the *Frt-neo-Frt-loxP* template [15]; distal *loxP* site in intron 6 is located 316 bp downstream from end of exon 6. *Third line*, *Slc39a8(f)* floxed allele in which *neo* was removed by FLP recombinase in mouse via breeding with FLP-transgenic mice. *Bottom*, *Slc39a8(−)* knockout allele following Cre recombinase-mediated excision, detected in MFF cultures (*see text*). (B) Southern blot, digestion by *Sca*I, confirming the *Slc39a8(t)* targeted allele (3017 bp) as distinct from the *Slc39a8(+)* wild-type allele (5072 bp).

2.2. Animals

For this study, the *Slc39a8(neo)* allele was in the mixed C57BL/6J (B6) and 129S6/SvEvTac background. All mouse experiments were conducted in accordance with the National Institutes of Health standards for the care and use of experimental animals and the University Cincinnati Medical Center Institutional Animal Care and Use Committee.

For in utero experiments, the morning on which the vaginal plug was found is considered gestational day-0.5 (GD0.5). *Slc39a8(+/+)*, *Slc39a8(+/neo)* and *Slc39a8(neo/neo)* embryos/fetuses, placentas and visceral yolk sacs were collected and genotyped at GD11.5, GD13.5 and GD16.5 for RNA isolation.

2.3. Total RNA preparation

Using Tri-Reagent (TR18, Molecular Research Center, Inc.; Cincinnati, OH), we isolated total RNA (combining those of the same genotype from one litter) from embryos, fetuses and yolk sacs; in each case, $N = 3$ or more pups.

2.4. Reverse transcription and quantitative real-time PCR analysis

Total RNA (2.5 μg) from embryos, fetuses, placenta or yolk sac – was used as a template for reverse transcription and primed with oligo(dT), using the SuperScript III first-strand kit, following the manufacturer's recommendations (Invitrogen). Total RNA (2.5 μg) was added to reactions containing 3.8 μM oligo$(dT)_{20}$ and 0.77 mM dNTP, to a final volume of 13 μL. Reactions were incubated at 65 °C for 5 min, then 4 °C for 2 min. Next, we added 7 μL of a solution containing 14 mM dithiothreitol and 40 units of RNaseOUT Recombinant RNase inhibitor™ (Verso cDNA kit, AB-1453/B, Thermo Scientific; Waltham, MA). After incubation at room temperature for 2 min, 1 μL of RT enzyme was added to each sample. Reaction tubes were incubated at 4 °C for 30 min, followed by 75 °C for 10 min (to inactivate reverse transcriptase) and placed immediately on ice. Diethylpyrocarbonate (DEPC)-treated distilled water (80 μL) was added to dilute the cDNA that had been generated, and the resultant mixture was stored at −80 °C until use.

We performed qRT-PCR in the Bio-Rad DNA Engine Opticon 2™ (Bio-Rad Laboratories; Hercules, CA), using iQ SYBR Green Super-