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Research paper

Expression of mouse *Dab2ip* transcript variants and gene methylation during brain development

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ABSTRACT

Dab2ip (DOC-2/DAB2 interacting protein) is a RasGAP protein which shows a growth-inhibitory effect in human prostate cancer cell lines. Recent studies have shown that Dab2ip also plays an important role in regulating dendrite development and neuronal migration during brain development. In this study, we provide a more complete description of the mouse *Dab2ip* (*mDab2ip*) gene locus and examined DNA methylation and expression of *Dab2ip* during cerebellar development. Analysis of cDNA sequences in public databases revealed a total of 20 possible exons for *mDab2ip* gene, spanning over 172 kb. Using Cap Analysis of Gene Expression (CAGE) data available through FANTOM5 project, we deduced five different transcription start sites for *mDab2ip*. Here, we characterized three different *mDab2ip* transcript variants beginning with exon 1. These transcripts varied by the presence or absence of exons 3 and 5, which encode a putative nuclear localization signal and the N-terminal region of a PH-domain, respectively. The 5′ region of the *mDab2ip* gene contains three putative CpG islands (CpG131, CpG54, and CpG85). Interestingly, CpG54 and CpG85 are localized on exons 3 and 5. Bisulfate DNA sequencing showed that methylation level of CpG54 remained constant whereas methylation of CpG85 increased during cerebellar development. Real-time PCR analysis showed that the proportion of PH-domain containing *mDab2ip* transcripts increased during cerebellar development, in correlation with the increase in CpG85 methylation. These data suggest that site-specific methylation of *mDab2ip* gene during cerebellar development may play a role in inclusion of exon 5, resulting in a Dab2ip transcript variant that encodes a full pleckstrin homology (PH) domain.

1. Introduction

Disabled homolog 2 interacting protein (Dab2ip), also known as apoptosis signal-regulating kinase 1 (ASK1)-interacting protein (AIP1) (Chen et al., 2002; Wang et al., 2002; Xie et al., 2009; Zhang et al., 2003), is a member of Ras GTPase-activating protein family (Wang et al., 2002; Zhang et al., 2003). Dab2ip is thought to be a tumor suppressor protein, regulating epithelial to mesenchymal transition (Min et al., 2010; Xie et al., 2010) and stem cell differentiation (Chang et al., 2013). In addition, down regulation of Dab2ip using shRNA enhances radio-resistance and proliferation in a metastatic prostate cancer cell line (Kong et al., 2010). Dab2ip also interacts with Dab1, a cytosolic adapter protein that controls neuronal migration and position during development. Dab2ip is widely expressed in specific regions of embryonic and adult mouse brain (Homayouni et al., 2003). Recent studies show mDab2ip regulates dendritic development and synapse formation in developing cerebellum (Qiao et al., 2013) and also plays crucial roles in neuronal migration in the developing neocortex (Lee et al., 2012; Qiao et al., 2015).

The *mDab2ip* gene is located on chromosome 2 (Chen et al., 2006). Originally, Chen et al. reported that *mDab2ip* spans 65 kb and contains 14 exons with one transcription start site (TSS) located on exon 1 (Chen et al., 2006). In addition, they showed that internal splice sites within exon 1 result in three variants, Ia, Ib and Ic. Since this initial report, several more Dab2ip cDNAs have been isolated from mouse and other species that contain additional 5′ exons. For example, the additional exons in human *Dab2ip* ortholog AIP1 (Acc# AY032952) encodes a complete pleckstrin homology (PH) domain (Von Bergh et al., 2004). Similarly,

Abbreviations: Aa, amino acid; cDNA, DNA complementary to RNA; CNS, central nervous system; DMSO, dimethylsulfoxide; dNTP, deoxyribonucleoside triphosphate; Dab2ip, disabled homolog 2 interacting protein; Bp, base pair; Kb, kilobase; mDab2ip, mouse Dab2ip; hDab2ip, human Dab2ip; AIP1, (ASK1)-interacting protein; TSS, transcription start site; PH domain, pleckstrin homology domain; C2 domain, PKC conserved 2; GRD, GAP-related domain; SMH, serine–methionine–histidine; CAGE, Cap Analysis of Gene Expression; CCD, Conserved Domain Database; BSP, bisulfite specific primers; MSP, methyl specific primers; P, postnatal day; 5′ (five prime), denotes a truncated gene at the 5′ end; 3′ (five prime), denotes a truncated gene at the 3′ end; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; PtdIns (3,4,5)P3, phosphatidylinositol (3,4,5)-triphosphate; PCs, Purkinje Cells; GCs, granule cells; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; qRT-PCR, quantitative reverse transcription-PCR; NCBI, National Center for Biotechnology Information; UCSC, University of California, Santa Cruz.

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our group identified a longer *mDab2ip* cDNA (Acc# DQ473307), which encodes a complete N-terminal PH-domain (Homayouni et al., 2003). Notably, both of these clones encode a protein with a shorter C-terminal region, ending with the amino acid sequence SMH (serine–methionine–histidine), which may function as a PDZ-interacting site.

The promoter region of both mouse and human *Dab2ip* gene is GC-rich (Chen et al., 2003, 2006). Several groups have shown that *hDab2ip* promoter is hyper-methylated in cancer cell lines and tumor tissues including prostate, breast, gastrointestine, lung and liver (Chen et al., 2003; Dote et al., 2004; Yano et al., 2005,; Qiu et al., 2007). In addition, both *hDab2ip* and *mDab2ip* promoters have been shown to be regulated by histone modification (Chen et al., 2002, 2003, 2006). Accumulating evidence suggests that promoter methylation plays a critical role during differentiation and maturation of the mammalian central nervous system (CNS) (Moore et al., 2012). To date, the methylation of *Dab2ip* promoter during development has not been investigated.

In the present study, we performed a thorough analysis of *Dab2ip* genomic organization using a variety of bioinformatic resources. We found at least five transcription start sites (TSS) and multiple putative translation initiation sites for *mDab2ip*. In addition, we identified four additional 5′ exons for *mDab2ip* than previously reported by Chen et al. (2006). We examined the expression of three *Dab2ip* 5′-splice variants, originating with the first TSS in exon one. Notably, *mDab2ip* contains three CpG islands located within exons 2, 3 and 5. Exon 5 CpG island was previously studied by Chen and colleagues and was shown to be the site of epigenetic control of *mDab2ip* expression (Chen et al., 2006). Here we show that methylation of exon 5 CpG (CpG85) increases during cerebellar development and is positively correlated with the inclusion of exon 5 in *mDab2ip* transcripts.

2. Materials and methods

2.1. Bioinformatic analysis

We deduced the exon–intron junction of *mDab2ip* by aligning its genomic DNA sequence with hDab2ip cDNA clone (Acc# NP-115941). The *mDab2ip* gene contains 20 exons and 19 introns (Supplementary Table 1). Transcription start site analysis was performed using Zenbu visualization tool and mouse Cap Analysis of Gene Expression (CAGE) data available through FANTOM5 project website (http://fantom.gsc.riken.jp/zenbu/gLyphs/#config=FP3UWqGsJVPtWNfSfupRk;loc=mm9::chr2:35370843..35629647+) (Forrest et al., 2014; Severin et al., 2014). Dab2ip protein motifs were examined using Conserved Domain Database (CCD) at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and MyHits protein motif search tool (Pagni et al., 2007).

2.2. Reverse transcription PCR

Based on the sequence information acquired from UCSC genome browser (mouse genome assembly mm9, July 2007), PCR primers were designed against specific 5′ exons of *mDab2ip* (Table 1). Total RNA was extracted from mouse cerebellum at different ages (P8, P14, P21 and P30) using TRIzol reagent (Invitrogen). cDNA was synthesized using the Transcriptor First Strand cDNA Synthesis Kit (Roche) with 1 μg of total RNA and oligo-dT primer. One-tenth volume of the first-strand reaction was used as a template for PCR amplification using the TaKaRa EX-Taq polymerase (Clontech Laboratories) and the primers listed in Table 1. Thermal cycling program started with an initial denaturation at 95 °C for 5 min, followed by 35 cycles (94 °C, 1 min; 61 °C, 1 min; 72 °C, 50 s) of amplification, followed by a 5 min extension at 72 °C.

2.3. Quantitative real-time PCR

Total RNA was isolated from mouse cerebellum at postnatal days 8, 14, 21 and 30 (P8, P14, P21 and P30) using TRIzol reagent (Invitrogen).

Table 1
List of oligonucleotide primers and TaqMan probe sequences used in this study.

Name	Sequence	Product size
Primers used for Dab2ip methylation		
CpG 131/MSP/M/F	5′-CGTTTTTTTTCGGTTTTAAACGTTTTTA-3′	243
CpG 131/MSP// M/R	5′-CGCCCTACAAACATCGTTCCCCGCGCGCT-3′	243
CpG 131/MSP / UM/F	5′-TGTTTTTTTTTGGTTTTAAATGTTTTTA-3′	243
CpG 131/MSP/ UM/R	5′-CACCCTACAAACATCATTCCCCACACACT-3′	243
CpG 131/BSP/F	5′-GAATTTGGGGAATATGGAGTAGAATAG-3′	264
CpG 131/BSP/R	5′-TTCCCTACCTTCTTTACTATAACCCAA-3′	264
CpG 54/MSP/M/F	5′-TTAGGTTAGGATTTTGTTCGTATTGATTTTTC-3′	303
CpG 54/MSP/M/R	5′-CTCCCGATACTCTTCCTAACGTTACCGCCGAC-3′	303
CpG 54/MSP/UM/F	5′-TTAGGTTAGGATTTTGTTTGTATTGATTTTTT-3′	303
CpG 54/MSP/UM/R	5′-CTCCCAATACTCTTCCTAACATTACCACCAAC-3′	303
CpG 54/BSP/F	5′-GTTTAGATATGGTTGTTGGGTATATGTT-3′	243
CpG 54/BSP/R	5′-AAAACCAAAACTACCCTACAAAATAACT-3′	243
CpG 85/MSP/M/F	5′-GAGTTTTTCGTTGTTCGATATAAAAGGTATTTTC-3′	153
CpG 85/MSP/M/R	5′-CGAATCTTAAATTATACCCATTAACCGAACGC CT-3′	153
CpG 85/MSP/UM/F	5′-GAGTTTTTTGTTGTTTGATATAAAAGGTATTTTT-3′	153
CpG 85/MSP/UM/R	5′-CAAATCTTAAATTATACCCATTAACCAAACACCT-3′	153
CpG 85/BSP/F	5′-GAGGTGGGTATTGTTTTTTGAGTAG-3′	193
CpG 85/BSP/R	5′-TACTCCTCCCCTCCAAATATTC-3′	193
Primers used for Dab2ip RT-PCR		
E1-53.84-F	5′-CGCTCATGGAGACGGCCTCGGTTCATAAATCA-3′	
E7-117.149-R	5′-AGCAGTAGTCCTGACCCAGGATGCTGCTGT GAA-3′	
Primers used for qRT-PCR		
GRD domain-F	5′-GCCTTCTGCAAGATCATCAAC-3′	
GRD domain-R	5′-GCTGATGAGCCGTTCACTG-3′	
PH domain-F	5′-CGCGGACAATGAGAGGTC-3′	
PH domain-R	5′-GAGCAGGGACTCGTGTGAC-3′	

MSP, methyl specific primer; BSP, bisulfite specific primer; F, forward; R, reverse.

Quantitative RT-PCR was performed on a LightCycler 480 Real-Time PCR System (Roche) using primers shown in Table 1 and Fig. 1A. Thermal cycling program started with an initial denaturation at 95 °C for 5 min, followed by 50 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 10 s, and final cooling at 40 °C for 10 s. The product sizes were confirmed by agarose gel electrophoresis, and melting curves were analyzed to control the specificity of PCR reactions. Dab2ip expression levels were normalized to β-actin. The relative levels of *Dab2ip* expression were measured by a modified ΔΔCt (Pfaffl, 2001). A two-tailed Student's *t*-test (unequal variance) was used to assess the significance of the change in three independent experiments.

2.4. DNA purification and bisulphite modification

Genomic DNA was isolated from snap frozen mouse cerebella collected at different time points (P8, P14, P21 and P30) using DNA purification kit (Qiagen) according to the manufacturer's instructions. Then, 500 ng of genomic DNA was denatured and treated with bisulfite, which converts all unmethylated cytosines to uracils without affecting methylated cytosine residues, using EZ DNA methylation-Gold kit (Zymo research) as described by manufacturer. The quality of bisulfite treatment was checked by measuring OD at 260/280 nm. The methylation status of each CpG island was analyzed separately by using bisulfite specific and methylation specific PCR. Bisulfite specific (BSP) and methyl specific (MSP) primers listed in Table 1, were designed using Methyl Primer Express Software v1.0 (Applied Biosystems), with some modifications (Table 1). Methyl specific PCR was performed by using two sets of primers for each CpG island. Each set of primers were able to distinguish between methylated (MSP/M) and unmethylated (MSP/UM) DNA sequence. PCR was carried out with approximately 30 ng of bisulfate treated genomic DNA in 30 μL total volume reaction containing 2.5 μl DMSO, 2.5 mM dNTP, and 0.20 U of TaKaRa EX-*Taq* DNA polymerase