J Hum Genet (1998) 43:123–127

ORIGINAL ARTICLE

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Identification of brain-specific splicing variants of the *hDLG1* gene and altered splicing in neuroblastoma cell lines

Received: October 30, 1997 / Accepted: November 27, 1997

Abstract The human homologue of *Drosophila* tumor suppressor dlg, hDLG1, is one of the proteins known to interact with APC, a tumor suppressor for colorectal cancer. Alternative splicing of this gene generates transcripts either with [insertion 1 (I1)] or without 99 nucleotides in the 5′ part of the dlg homology repeats (DHR) domain. We found almost equivalent expression of these two splicing variants in most human tissues; however, in skeletal muscle the transcript with the 99-bp insertion was predominant, and in the brain, that without the 99-bp insertion was expressed predominantly. We also examined alternative splicing in the region between the SH3 and GUK domains where two different sizes of insertions, 34 nucleotides (I2) or 100 nucleotides (I3), had been reported, and found various splicing patterns among the tissues examined. In brain we detected six different, alternatively spliced transcripts, two of which included a novel, 36-bp, brain-specific exon encoding a peptide bearing significant homology to a portion of rat synapse-associated protein, SAP97/PSD95. Subsequently, we investigated the splicing patterns of the *hDLG1* gene in 24 neuroblastoma cell lines. In two-thirds of these lines, the splicing patterns were altered from those observed in normal brain tissue. As one-third retained the normal brain-splicing pattern, the loss of normal splicing of *hDLG1* may not in itself cause formation of tumors, but it might reflect the biological character of individual neuroblastomas.

Key words Splicing variants · *hDLG1* gene · Neuroblastoma

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Introduction

The *hDLG1* gene is a human homologue of *Drosophila* discs-large *(dlg)*, a gene that is essential for normal synapse formation and for structure of septate junctions in the fruit fly (Stewart et al. 1972; Abbott and Natzle 1992; Lahey et al. 1994; Lue et al. 1994). *Drosophila* dlg is characterized as a tumor suppressor because its dysfunction leads to overgrowth of imaginal discs, which lose their epithelial structure and their ability to differentiate (Woods and Bryant 1991; Woods et al. 1996). Although the tumor-suppressor function of hDLG1 has not been proven, its ability to bind adenomatous polyposis coli (APC), a known suppressor of colorectal tumors (Matsumine et al. 1996), suggests that it regulates APC function and plays an important role in the mechanism of tumor suppression involving APC.

hDLG1 contains three functionally important domains that are similar to those in *Drosophila dlg:* dlg homology repeats (DHR), an src homology-3 region motif (SH3), and a guanylate kinase (GUK) domain (Lue et al. 1994; Azim et al. 1995) (Fig. 1A). RNA transcripts of *hDLG1* are widely expressed in human tissues including the central nervous system and digestive tract. The gene is known to be spliced alternatively at two sites: one alternative splicing occurs in the 5′ part of the DHR domain and generates transcripts with or without a 99-nucleotide segment (Insertion 1; I1) (Lue et al. 1994); the other occurs between the SH3 and

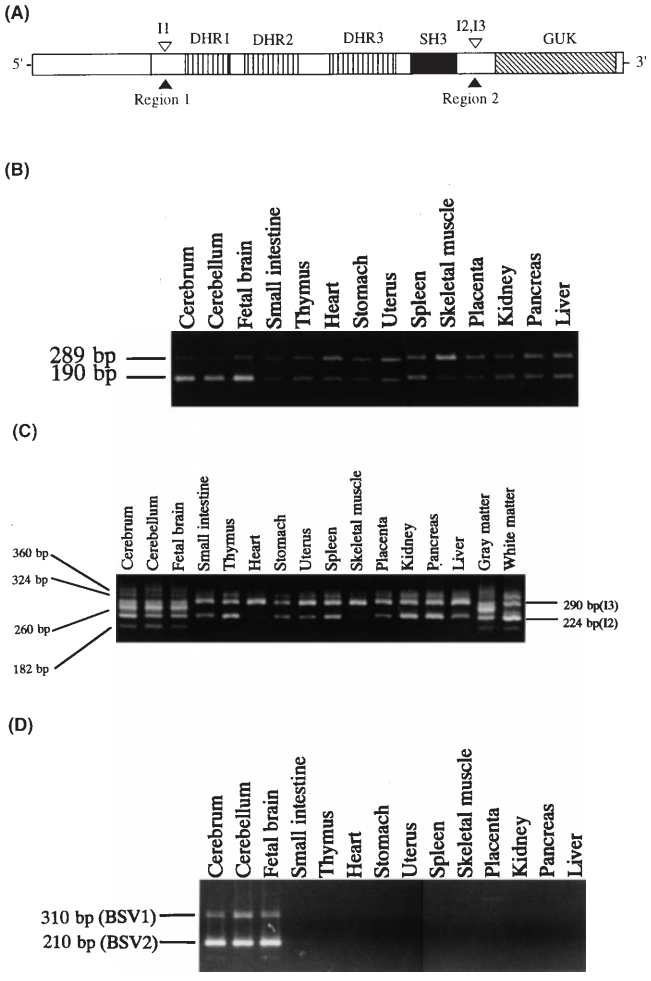

Fig. 1 **A** Schematic structure of the *hDLG1* gene. The *hDLG1* gene contains three known functional domains: DLG homology repeat (*DHR*), src homology 3 region motif (*SH3*), and guanylate kinase domain (*GUK*). Two alternative splicing sites are known. One occurs at region 1, yielding products with or without Insertion 1 (*I1*); the other occurs at region 2, where Insertion 2 (*I2*) or Insertion 3 (*I3*) are transcribed. **B** Reverse transcriptase polymerase chain reaction (RT-PCR) of region 1. *hDLG1* transcripts are expressed in all tissues examined. Fragments of 289 bp and 190 bp correspond to PCR products with Insertion 1 (*I1*) and without I1, respectively. The transcript containing I1 is dominant in skeletal muscle, but in cerebrum, cerebellum, and fetal brain, transcription products containing I1 are very scarce. Other tissues express products with or without I1 almost equally. **C** RT-PCR of region 2. Fragments of 224 bp and 290 bp correspond to PCR products with *I2* and *I3* respectively. In most organs, I2 and I3 are almost equally prevalent, but in skeletal muscle and heart the transcript containing I2 is hardly detectable. In addition to I2 and I3, a faint Insertion 4 (I4) band (324 bp) is detectable in most of the tissues. Brain tissue shows a unique pattern of seven splicing variants, three of them brain-specific, although this pattern is not complete in white matter. **D** PCR using a primer specific to region D (primer pair 3, Table 1). Products appear only in lanes representing cerebrum, cerebellum, and fetal brain. Fragments of 310 bp and 210 bp correspond to BSV1 and BSV2, respectively

GUK domains, where two different sizes of insertions, 34 nucleotides (I2) or 100 nucleotides (I3), have been reported (Lue et al. 1994). However, any differences in the physiological functions of proteins generated by these splicing variants, or in expression patterns in various tissues, have yet to be described.

To investigate the regulatory mechanism of *hDLG1* expression and its possible role in carcinogenesis, we examined expression patterns of its transcripts in various human tissues and in 26 cell lines derived from neural tumors. Here, we report identification of novel splicing variants, some of which were transcribed in a tissue-specific manner, and alteration of splicing patterns in some neuroblastoma cell lines.

Materials and methods

Cell lines and polyA RNAs

Glioblastoma cell lines U373MG and A172 were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). The 24 neuroblastoma cell lines for our experiments, SK-N-SH, SK-N-AS, SK-N-BE, SK-N-DZ, SH-SY-5Y, RTBM-1, LA-N-5, IMR-32, NMB, NGP, NLF, OAN, NBL-S, GAMB, SMS-KAN, SMS-SAN, LHN, NB1, SMS-KCN, CHP901, TGW, NBTU1, CHP134, and NBKM1, were described previously (Azar et al. 1990; Nakagawara et al. 1994). Total RNAs were isolated with Trizol Reagent (Gibco BRL, Gaithersburg, MD, USA) from each of these cell lines as well as from white matter and gray matter of cerebrum that was obtained with informed consent from a surgical specimen of a 25-year-old male patient. PolyA RNA preparations derived from cerebrum, cerebellum, fetal brain, small intestine, thymus, heart, stomach, uterus, spleen, skeletal muscle, placenta, kidney, pancreas, and liver were purchased from Clontech Laboratories, Palo Alto, CA, USA.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

One microgram of polyA RNA from each of a variety of human tissues and 3 μg of total RNAs from each of the cancer-cell lines were reversely transcribed for single-strand cDNA using oligo$(dT)_{15}$ primer and SuperscriptII (Gibco BRL). Each single-stranded cDNA was diluted for subsequent PCR reactions by monitoring the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a quantitative control. PCR was carried out in a 20-μl reaction mixture at 94°C for 4 min for initial denaturing, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s on the GeneAmp PCR system 9600 (Perkin Elmer, Norwalk, CT, USA). The primer pairs to amplify each region of *hDLG1* are listed in Table 1. The PCR products were separated by electrophoresis in 2% or 3% agarose gels.

Cloning and sequencing of splice variants

The PCR products corresponding to splicing variants were separated in agarose gels. Each fragment was recovered from the gel, purified using Geneclean II (BIO 101, La

Table 1 Primers used for RT-PCR

Forward primers	Reverse primers
Region 1	
Pair 1:5′-GTATCAGGATGAAGATACACCT-3′	5′-ATCTGTGTTGACCAGTACTGG-3′
Region 2	
Pair 2:5′-GTTGAGAAGAAAGAACGAGCCCG-3′	5′-CCCAATATGATCACTGGTCGAGT-3′
Pair 3:5′-GAGAGCGATGAGGTCGGAGTG-3′	5′-GCAGCCATACTCATCTGTAATC-3′

Jolla, CA, USA), subcloned into pBluescriptII SK(−) vector (Stratagene, La Jolla, CA, USA), and sequenced by means of the Dye Terminator Cycle Sequencing FS Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and an ABI 373 DNA automated sequencer (Perkin Elmer), using T3 or T7 primers.

Results

Tissue distributions of *hDLG1* splicing-variants

We performed RT-PCR experiments using cDNAs synthesized from polyA RNAs derived from various organs as templates, to investigate alternative splicing of the *hDLG1* gene. When we examined the 5′ part of the DHR domain (region 1), where a known alternative-splicing event results in an insertion (I1) or an absence of 99 nucleotides, the two transcripts were almost equivalently observed in all human tissues examined except for skeletal muscle and brain; in skeletal muscle, the transcript with the 99-bp insertion was expressed predominantly, but in cerebrum, cerebellum, or fetal brain, the transcript with the 99-bp insertion was hardly detectable (Fig. 1B).

We then investigated alternative-splicing patterns in the region between the SH3 and GUK domains (region 2), where two major splice-forms, Insertion 2 (I2; 34 nucleotides) or Insertion 3 (I3; 100 nucleotides), had already been reported (Lue et al. 1994). PCR amplification of this region using cDNAs from various human tissues revealed a complex pattern of alternative splicing (Fig. 1C). In most tissues a previously unreported transcript (Insertion 4; I4) of 324 bp, larger than I3, was detectable. Thymus, spleen, kidney, and pancreas expressed both I2 and I3 transcripts almost equivalently while the I3 transcript seemed to be more predominant in small intestine, stomach, uterus, placenta, and liver. The I2 transcript in skeletal muscle and heart is hardly detectable, indicating that these two tissues seemed to express the transcript I2 significantly less abundantly. Moreover, in brain, three different sizes of bands in addition to the I2, I3, and I4 transcripts were detected. Among the three brain-specific variants, one was smaller than the I2 transcript, one was larger than the I3 transcript, and one was seen between the I2 and I3 transcripts. To examine whether the bands specifically observed in brain had originated from neuronal cells or glial cells, we performed PCR using cDNAs from white matter and gray matter. All three brain-specific splicing variants were observed when RNA from gray matter was used as a template, an indication that the novel forms were generated mainly in neuronal cells.

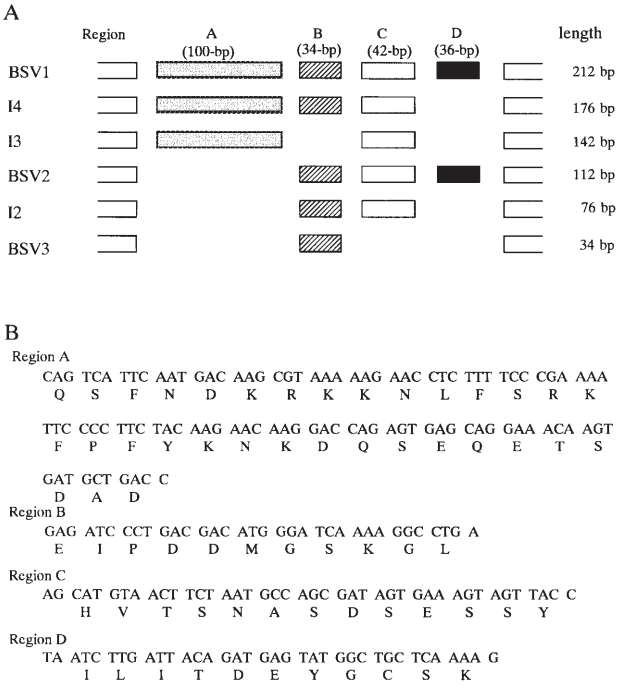

Fig. 2 **A** Splicing variants at region 2 and their components. Brain-specific variants (*BSV*) 1–3 and Insertion 4 (*I4*) are novel splicing variants; furthermore, region *D* is a heretofore unreported exon that is expressed only in brain. As regions *A* and *B* are transcribed simultaneously in BSV1 and I4, a stop codon appears in region *B* and produces truncated proteins. **B** Nucleotide (*above*) and amino acid sequence (*below*) of each exon. The amino acid sequence of region *D* bears a high degree of homology to the amino acid sequence of SAP97/PSD95, the rat homologue of dlg

Characterization of alternatively-spliced variants in brain

To further characterize the various-sized PCR products in brain, we subcloned each of the six PCR fragments and determined their DNA sequences. Transcripts I2, I3, and I4 consisted of the exons indicated as regions B and C (I2); A and C (I3); and A, B, and C (I4) in Fig. 2. DNA sequencing analyses also disclosed a novel, 36-bp exon (D) distal to region C. The three brain-specific transcripts were generated by various combinations of exons A, B, C, and D. The largest transcript, termed brain-specific variant 1 (BSV1), contained all four exons; BSV2 contained three; and BSV3

contained only exon B in their transcripts. Comparisons with the I2 and I3 transcripts which are known to contain appropriate open reading frames suggested that BSV1 and I4 would cause frame-shifts resulting in premature termination of the gene products. The other two brain-specific transcripts, BSV2 and BSV3, are in-frame, although they encode peptides different from the hDLG1 reported originally; BSV2 includes an additional 12 amino acids distal to region C, and BSV3 would lack a peptide corresponding to region C.

To confirm that region D is transcribed specifically in the brain, we performed PCR using an oligonucleotide corresponding to region D as one of the PCR primers. As shown in Fig. 1D, the RT-PCR product including region D was detectable only in lanes where the templates were cDNA from adult or fetal brain.

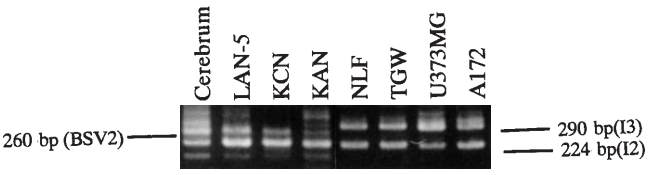

Fig. 3 Electrophoresis of PCR products at region 2. RT-PCR products of five neuroblastoma cell lines (LA-N-5, SMS-KCN, SMS-KAN, NLF, and TGW), two glioblastoma cell lines (U373MG and A172), and normal cerebrum are shown. PCR products of 290-bp, 260-bp, and 224-bp correspond to I3, BSV2, and I2 respectively. LA-N-5 and SMS-KCN expressed abundant BSV2, which contains the product of the brain-specific exon D. NLF and TGW demonstrated the same expression pattern as U373MG and A172: none of these transcribed BSV2. SMS-KAN showed an expression pattern intermediate between those of these two groups; both BSV2 and I3 were transcribed at very low levels

Variable splicing patterns in neuroblastoma cell lines

We assumed that the brain-specific transcripts and their peptide products might contribute to brain-specific function or growth regulation. To investigate this hypothesis, we examined expression of the *hDLG1* gene in cell lines derived from 24 neuroblastomas and two glioblastomas. RT-PCR experiments revealed that nine of the neuroblastoma cell lines (SK-N-SH, RTBM-1, LA-N-5, IMR-32, NGP, GAMB, SMS-SAN, LHN, and SMS-KCN) abundantly expressed BSV2, the variant that contains the brain-specific 36-bp exon (exon D). Furthermore, in SMS-KCN and LHN, the transcript corresponding to I3 was significantly reduced. Eight other neuroblastoma cell lines (SK-N-AS, SK-N-BE, SK-N-DZ, NMB, NLF, NB1, TGW, and NBKM1) as well as the glioblastoma cell lines (U373MG and A172), revealed expression patterns similar to most of the other tissues and failed to express the BSV2 form. In the remaining seven neuroblastoma cell lines we examined (SH-SY-5Y, CHP134, OAN, NBL-S, SMS-KAN, CHP901, and NBTU1), expression of both BSV2 and I3 was significantly reduced. These results led us to classify the neuroblastoma cell lines into three groups on the basis of their patterns of BSV2 and I3 expression. Figure 3 shows representative RT-PCR patterns of each of the three groups.

Discussion

Drosophila dlg is localized in the septate junctions that connect all imaginal-disc epithelial cells, and in the fly this protein probably plays an important role in regulation of cell polarity and proliferation (Woods and Bryant 1991). Its dysfunction leads to abnormal structure of junctions and loss of cell polarity, and induces neoplastic proliferation. The human homologue of dlg, hDLG1, belongs to the family of proteins termed membrane-associated guanylate kinase homologues (MAGUKs) that include postsynaptic density protein PSD95/SAP90 and tight junction proteins ZO-1 and ZO-2 (Azim et al. 1995). The hDLG1 protein contains three recognized, biologically important domains conserved from *Drosophila*; the first is characterized by three DHR segments within the N-terminal region, each of which is composed of 90 amino acids (Lue et al. 1994). The recent finding that hDLG1 binds to APC, a known suppressor of colorectal tumors, through this DHR domain, suggested that hDLG1 may function as a tumor suppressor in humans by regulating APC (Matsumine et al. 1996). The second functional domain, SH3, is present in many proteins that play significant roles in membrane-associated signal transduction pathways, and is considered to mediate binding to other proteins (Ren et al. 1993). The third important region, the GUK (guanylate kinase) domain, lies in the C-terminal portion of hDLG1. Since mutations in the GUK region of *Drosophila* dlg are associated with neoplastic proliferation, this domain is likely to have a tumor-suppressive function in humans as well (Woods and Bryant 1991; Woods et al. 1996).

In this report, we have demonstrated tissue specificity in alternative splicing of the *hDLG1* gene. Alternative splicing in the 5′ portion results in the presence or absence of a 33-amino-acid, proline-rich peptide similar to the SH3-binding consensus sequence. The major transcript in the brain lacks this peptide, but skeletal muscle expresses the transcript with those 33 amino acids more abundantly than it expresses the shorter variant. One member of the MAGUKs family of molecules, ZO-1, with a similar proline-rich domain, is also alternatively spliced in this domain (Willott et al. 1993); the isoform containing the proline-rich domain is detected in most epithelial cell junctions while the other is expressed in endothelial cells or only in specific epithelial junctions. Although the physiological roles of the proteins encoded by these splicing variants are not well understood, it is likely that the variant proteins are significantly associated with tissue-specific roles of both hDLG1 and ZO-1.

The second region of *hDLG1* subject to alternative splicing lies between the SH3 and GUK domains. In addition to the two alternatively spliced transcripts reported previously, we found four additional transcripts. Among the four newly identified transcripts, three were brain-specific, and two of those contained the heretofore unreported exon in-

dicated as region D in Fig. 2. Seven of the 12 amino acids encoded by region D are identical to part of synapse-associated protein SAP97/PSD95, the rat homologue of *Drosophila* dlg. As this portion was transcribed only in the brain, this conserved region is likely to have a significant function there.

The major I2 and I3 forms reported previously and a novel, minor transcript (I4) were detected in most tissues, although the I2 transcript was much less abundant in skeletal muscle and heart. We found a more complicated splicing pattern of the *hDLG1* gene in the brain, however, where we detected at least six transcripts (Fig. 2); the I2, BSV2, and I3 forms were the most abundant, but three minor forms, BSV1, BSV3, and I4, were detected also. As the BSV1 and I4 splices probably cause a shift of reading frame, and because western blotting analyses of hDLG1 protein failed to recognize their truncated products (data not shown), these two transcripts are likely to be nonfunctional, although we have no conclusive evidence for that.

On the basis of these observations, we assumed that the brain-specific exon (D) and the brain-specific alternative splicing pattern could be associated with a significant physiological function of hDLG1 in the brain. As alternative splicing is known to play a crucial role in tumor development and/or progression in some cancer tissues (Tempfer et al. 1996), we investigated the splicing pattern of *hDLG1* in 24 neuroblastoma cell lines and two glioblastoma cell lines. We found that the splicing patterns of *hDLG1* in about two-thirds of the 24 neuroblastoma cell lines examined were different from that of normal brain. However, as nearly one-third of the neuroblastoma lines did retain the normal splicing pattern, the loss of normal splicing may not be the cause of tumor development; rather, it might influence the biological character of individual neuroblastomas. Nevertheless, the findings reported here should contribute to an eventual understanding of the tissue-specific functions of hDLG1 and regulation of its expression.

Acknowledgments This work was supported in part by a special grant for Strategic Advanced Research on Cancer from the Ministry of Education, Culture, Sports, and Science of Japan; and a "Research for the Future" Program Grant (96L00102) of The Japan Society for the Promotion of Science.

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