Commisural neurons in the spinal cord project their axons through the floor plate using a number of molecular interactions, such as netrins and their receptor DCC (deleted in colorectal cancer). However, the molecular cascades that control differentiation of commissural neurons are less characterized. A homeobox gene, MBH1 (mammalian BarH1) was expressed specifically in a subset of dorsal cells in the developing spinal cord. Transgenic mice that carried lacZ and MBH1-flanking genome sequences demonstrated that MBH1 was expressed by commissural neurons. To analyze the function of MBH1, we established an in vivo electroporation method for the transfer of DNA into the mouse spinal cord. Ectopic expression of MBH1 drove dorsal cells into the fate of commissural neurons with concomitant expression of TAG-1 (transiently expressed axonal surface glycoprotein 1) and DCC. Cells ectopically expressing MBH1 migrated to the deep dorsal horn, in which endogenous MBH1-positive cells accumulated. These results suggest that MBH1 functions upstream of TAG-1 and DCC and is involved in the fate determination of commissural neurons in the spinal cord.

Key words: MBH1; homeobox; homeodomain; in vivo electroporation; TAG-1; DCC

Introduction

Commisural neurons in both vertebrates and invertebrates transfer information from one side of their bodies to the other through the midline. Molecular mechanisms regulating axon guidance of these neurons have been characterized extensively (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Kaprielian et al., 2001). Netrins and DCC (deleted in colorectal cancer) play a pivotal role in axon guidance. Some commissural neurons are generated in the developing dorsal spinal cord, in which domains of progenitor cells are specified by helix-loop-helix (HLH) transcription factors (Gowan et al., 2001). The domains are initially established by TGF-β-like signals (Liem et al., 1997) and produce several cell types, which are defined by combinatorial expression of homeobox genes (Lee and Jessell, 1999; Gross et al., 2002; Muller et al., 2002). The most dorsal cell type, dI1 (D1), is generated by an HLH factor, MATH1 (mouse atonal homolog 1). dI1 cells and a subset of commissural neurons are lost in MATH1 knock-out mice (Bermingham et al., 2001; Gowan et al., 2001), whereas ectopic expression of MATH1 increases the number of dI1 cells (Gowan et al., 2001). However, the molecular cascades that form the link between the generation of the cells and their migration–axon guidance remain to be determined.

Bar-class homeobox (BarH) genes function in the development of various organs. Drosophila BarH genes control the development of the retina (Higashijima et al., 1992a) and peripheral nervous system (Higashijima et al., 1992b). A mammalian BarH gene, MBH1, is expressed at early stages of neurogenesis and is a potential regulator of neural HLH genes in the diencephalon (Saito et al., 1998). Outside of the diencephalon, MBH1 is expressed by postmitotic neurons in the midbrain, hindbrain, spinal cord, and retina (Saito et al., 1998, 2000). Another mammalian BarH gene, MBH2/BarH1, is also expressed in the spinal cord (Bullone et al., 2000; Saito et al., 2000) and suggested to be a downstream gene of MATH1 (Bermingham et al., 2001). Xenopus BarH genes, XBH1 and XBH2, which are orthologs of MBH1 and MBH2, respectively, show distinct expression patterns (Patterson et al., 2000). Expression patterns of MBH1 and MBH2 are similar but not identical (Saito et al., 2000), suggesting that expression of the two genes may be controlled by different mechanisms.

In this paper, we made transgenic mice carrying lacZ with the MBH1-flanking sequences and examined the cell types of MBH1-expressing cells in the developing mouse spinal cord. The function of MBH1 has been revealed using the in vivo electroporation method.

Materials and Methods

Generation and analysis of transgenic mice. The MBH1-flanking sequences were obtained by screening a 129/SvJ mouse genomic library (Stratagene, La Jolla, CA) using the entire sequence of the MBH1 cDNA (GenBank accession number AB004056) as a probe. We made a construct that carried the lacZ-coding region from BGZA (Yee and Rigby, 1993; Helms et al., 2000) between the 1 kb 5’ and 2.5 kb 3’ sequences flanking the MBH1-coding region. BGZA was a gift from Dr. J. Johnson.
Results

**MBH1 expression marked a subset of cells in the spinal cord**

Expression of MBH1 was detected in the mouse dorsal spinal cord at embryonic day 10.5 (E10.5) (Fig. 1A). At E11.5, the expression expanded ventrally to the deep dorsal horn (Fig. 1C). Later than E12.5, the expression was mainly restricted to the deep dorsal horn (Fig. 1E). The pattern of the expression during development resembled that of ventral migration of some dorsal neurons (Leber and Sanes, 1995), suggesting that MBH1 was expressed by these migrating neurons. A stream of cells between the deep dorsal horn and the floor plate also expressed MBH1 at E12.5 (Fig. 1E, arrows). MBH1 expression in the ventral spinal cord became confined to a group of cells dorsolateral to the floor plate at later stages (see Fig. 4B), suggesting that MBH1 + cells in the stream are under ventromedial migration at E12.5.

**Characterization of MBH1-expressing cells**

To examine which types of cells expressed MBH1, transgenic mice with lacZ under the control of the MBH1-flanking DNA sequences were generated. The transgenic mice expressed the lacZ product β-gal in a pattern recapitulating endogenous MBH1 expression (Fig. 1B, D, F). Coexpression of MBH1 and lacZ was confirmed by immunostaining using an anti-MBH1 antibody (data not shown). All β-gal + cells were labeled with antibodies against the LH2A/B proteins (Fig. 1G), a marker of d11 cells, but
not with antibodies against the Isl1 and Lim1/2 proteins (data not shown), suggesting that MBH1 is expressed by dI1 cells.

Because the β-gal protein spreads throughout the cytoplasm, it enabled us to examine the morphologies of MBH1− cells. β-gal− signals were detected in axons projecting to the floor plate and ventral funiculi (Fig. 1H). The β-gal− axons were labeled with specific markers of commissural neurons, anti-TAG-1 and anti-DCC antibodies (Fig. 1H; data not shown). These results indicate that MBH1 is expressed by commissural neurons.

**In vivo electroporation into the spinal cord**

To examine the function of MBH1, we established a system for the forced expression of a gene in the mouse spinal cord by modifying our *in vivo* electroporation method to the brain (Saito and Nakatsuji, 2001). The uterine wall was cut to see embryos clearly, and DNA was injected into the central canal of the spinal cord (Fig. 2). Then electric pulses were applied to the spinal cord using half-ring-type electrodes (Fig. 2A). The electrodes helped better survival of embryos by limiting electric pulses mainly onto the spinal cord. After electroporation, a reporter gene, EYFP, was expressed only in one side of the spinal cord (Fig. 2B, C).

**Ectopic expression of MBH1**

Using this system, pEYFP-MBH1, which carried both the EYFP and MBH1 genes downstream of ubiquitous CAG promoters, was introduced into the E11.5 mouse spinal cord. At this stage, DNA will be taken up by cells that are not fated to express MBH1 for the following two reasons: (1) endogenous MBH1+ cells are away from the ventricle (Fig. 1C), and (2) DNA is transferred to cells adjacent to the ventricle by this method (Saito and Nakatsuji, 2001). DNA will be also introduced mainly into dorsal cells, because the central canal is wider in the dorsal side. Transfection with EYFP alone as a control mostly labeled only dorsal cells, as expected (Fig. 3A). In contrast, more ventral EYFP+ cells were generated by coexpression with MBH1 (Fig. 3B, arrows). The ventral EYFP+ cells had morphologies similar to some commissural neurons (Silos-Santiago and Snider, 1992). Transfection of MBH1 also produced more EYFP+ commissural axons (Fig. 3B,
horn, in which the endogenous MBH1⁺ cells accumulated (Fig. 4B). A minor population of the MBH1-misexpressing cells was detected in the ventral spinal cord as well as the endogenous MBH1⁺ cells. These results suggest that the cells ectopically expressing MBH1 migrate to endogenous MBH1⁺ domains.

Next we examined whether the misexpression of MBH1 affects other genes. Expression of MATH1 and LH2B, which are related to the differentiation of commissural neurons, was not upregulated (Fig. 4C,D; data not shown). Furthermore, no increase of LH2A/B⁺ cells was detected using the anti-LH2A/B antibody (data not shown). These findings suggest that MATH1 and LH2A/B are not downstream of MBH1.

Discussion

Commissural neuron differentiation by MBH1

The results obtained by the ectopic expression of MBH1 suggest that MBH1 regulates at least three aspects of the differentiation of commissural neurons. Expression of TAG-1 and DCC, which are markers of commissural neurons, were induced by MBH1. Because DCC is a receptor of netrins, the ectopic expression of DCC may be responsible for axon projection of the MBH1-misexpressing cells to the floor plate. Their axons elongated along the floor plate after crossing it, as do endogenous commissural neurons. The MBH1-misexpressing cells appeared to migrate to the endogenous MBH1⁺ domains. These findings suggest that several genes involved in the differentiation of commissural neurons are regulated downstream of MBH1.

There are several types of commissural neurons at various dorsoventral domains in the spinal cord. Only two domains were MBH1⁺, showing that MBH1 is expressed by a subset of commissural neurons. The expression of MBH1 at E10.5, which was restricted to the dorsal edge of the spinal cord, was similar to that of MATH1, but the expression of MATH1 was limited to the ventricular zone and detected at E9.5 (Helms and Johnson, 1998; data not shown), earlier than that of MBH1. All β-gal⁺ cells of the MBH1/lacZ transgenic embryos expressed LH2A/B, which is a marker of dI1 cells and expressed downstream of MATH1. These results indicate that MBH1 is expressed in a lineage of cells that have expressed MATH1. All LH2A/B⁺ cells, however, appeared not to be β-gal⁺, suggesting that MBH1 is expressed in a subset of LH2A/B⁺ cells. The expression patterns of MBH1 were similar to those of LH2B in the spinal cord (Fig. 4; data not shown), suggesting that MBH1 may be expressed in the same lineage of cells that express LH2B.

Misexpression of MBH1 generated more commissural neurons without induction of LH2A/B, suggesting that LH2A/B may not exert the same function as MBH1 in the differentiation of commissural neurons. This was confirmed by ectopic expression of LH2B in the spinal cord, which did not produce more commissural neurons (data not shown). On the other hand, misexpression of MATH1 generated more commissural neurons (data not shown), suggesting that MATH1 is upstream of MBH1. At E11.5, the domain of MBH1 expression closely resembled the β-gal⁺ domain of transgenic mice that carried lacZ under the control of MATH1-flanking sequences (Helms and Johnson, 1998). Moreover, the 3’ MBH1-flanking sequence used for the transgenic mice in this study contained an E-box (CAGCTG), which could bind the MATH1 protein (Akazawa et al., 1995; Helms et al., 2000). These findings suggest that MBH1 may be a downstream target of the MATH1 protein.

A recent report has shown that excess commissural neurons were generated in Lbx1 (a Ladybird-like homeobox gene 1) mutant mice because of mis-specification of dorsal interneurons (Gross et al., 1999; Iwai et al., 2000). A minor population of the MBH1-misexpressing cells was detected in the ventral spinal cord as well as the endogenous MBH1⁺ cells. These results suggest that the cells ectopically expressing MBH1 migrate to endogenous MBH1⁺ domains.

Next we examined whether the misexpression of MBH1 affects other genes. Expression of MATH1 and LH2B, which are related to the differentiation of commissural neurons, was not upregulated (Fig. 4C,D; data not shown). Furthermore, no increase of LH2A/B⁺ cells was detected using the anti-LH2A/B antibody (data not shown). These findings suggest that MATH1 and LH2A/B are not downstream of MBH1.

Migration patterns of MBH1-misexpressing cells

Four days after electroporation, cells expressing EYFP alone remained in the dorsal spinal cord (Fig. 3I). In contrast, most of MBH1-misexpressing cells were observed in the middle of the spinal cord (Fig. 3J), suggesting that MBH1-misexpressing cells may have migrated from the dorsal spinal cord. To compare MBH1-misexpressing cells with endogenous MBH1⁺ cells, in situ hybridization was performed (Fig. 4). At E12.5, 1 d after electroporation, more cells expressing MBH1 were detected in the dorsal area of the MBH1-transfected side (Fig. 4A). Expression levels of MBH1 were higher in the MBH1-misexpressing cells than those of endogenous MBH1, reflecting a strong activity of the CAG promoter. Those cells appeared to migrate toward the deep dorsal horn, whereas the endogenous MBH1⁺ cells had already settled in the deep dorsal horn at this stage (Fig. 4A, arrowhead). Two days after electroporation, many MBH1-misexpressing cells settled down in the deep dorsal

Figure 4. Comparison of MBH1-misexpressing cells with endogenous MBH1⁺ cells using in situ hybridization. After electroporation at E11.5, transverse sections of the mouse spinal cord were annealed with antisense cRNA probes of MBH1 (A, B), MATH1 (C), and LH2B (D). Right sides were transfected. Embryos were recovered at E12.5 (A, C, D) and at E13.5 (B). Arrowheads and arrows indicate endogenous MBH1⁺ domains and MBH1-misexpressing cells, respectively. Expression of MATH1 and LH2B was not upregulated at E13.5 (data not shown). Transfection with EYFP alone did not affect the expression of the genes (data not shown). Scale bar (in A): A–D, 100 μm.
et al., 2002). This is similar to our results from the misexpression of MBH1. However, expression of Isl1 and Lim1/2, which are affected in the mutant mice, were not perturbed by the misexpression of MBH1 (data not shown). This result suggests that MBH1 generates ectopic commissural neurons independently of a transcriptional cascade exerted in the Lbx1 mutant mice.

**Regulation of cell migration by MBH1**

The transgenic mice carrying lacZ with the MBH1-flanking sequences visualized MBH1+ cells. At E10.5 and E11.5, the stages when the MBH1+ cells were located between the dorsal edge and the deep dorsal horn in the spinal cord, they showed morphologies typical of some migratory neurons (unipolar with leading processes) (Leber and Sanes, 1995). Together with expression patterns of MBH1, this suggests that MBH1 is expressed during the migration of commissural neurons. The endogenous MBH1+ cells migrated to the deep dorsal horn along the marginal zone of the spinal cord. In contrast, MBH1-misexpressing cells appeared not to follow the same route as the endogenous MBH1+ cells but rather to take a direct shortcut route from their birthplaces in the ventricular zone to the deep dorsal horn. These observations suggest that MBH1 may instruct the cells where to migrate, responding to an extracellular factor in the spinal cord. The factor may be released from the deep dorsal horn to attract the cells or may exclude the cells from the dorsolateral region of the spinal cord.

MBH1 was also expressed by granule cells during their migration in the developing cerebellum (Saito et al., 2000). MATH1, TAG-1, and DCC are all expressed in the developing cerebellum as well (Yamamoto et al., 1990; Akazawa et al., 1995; Livesey and Hunt, 1997), suggesting that there is a common cascade of genes between commissural neurons and the cerebellum.

**Various functions of BarH genes**

Some commissural neurons are generated downstream of Ngn2 (Simmons et al., 2001). We showed that forced expression of MBH1 upregulates Ngn2 in P19 cells, reflecting expression patterns of the two genes in the developing diencephalon (Saito et al., 1998). Ngn2 was not activated by ectopic expression of MBH1 in the developing spinal cord (data not shown). MBH1 requires another unknown factor that is transiently expressed in P19 cells to upregulate Ngn2 (Saito et al., 1998). The factor may have been absent in the spinal cord at the stage when MBH1 was ectopically expressed. MBH1 was expressed in mitotically active cells in the ventricular zone of the diencephalon, whereas postmitotic cells expressed MBH1 in the spinal cord. MBH1 may have different functions at different stages of neurogenesis. Similarly, Drosophila BarH genes show various functions (Higashijima et al., 1992a,b).

**In vivo electroporation in mouse**

Both gain- and loss-of-function analyses are essential to establish a gene function. Gene knock-out techniques have enabled the loss-of-function analysis of many genes in mouse. On the other hand, gain-of-function approaches have been used extensively in chick. The genes and gene combinations that regulate some stages of development are not exactly the same between chick and mouse. This report demonstrates that the *in vivo* electroporation technique is a powerful tool to reveal gene function in the mouse. This technique will greatly facilitate functional analyses of genes, because it may also be applied to knock-out and transgenic mice.

**References**


