A novel neural-specific BMP antagonist, Brorin-like, of the Chordin family

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abstract

We identified a gene encoding a novel secreted protein in mice, humans, and zebrafish. As the protein of 222 amino acids is similar to Brorin, a secreted BMP antagonist, which is a member of the Chordin family, we named it Brorin-like. Recombinant Brorin-like protein weakly but significantly inhibited the activity of BMP in mouse preosteoblastic cells and promoted neurogenesis in mouse neural precursor cells. Brorin-like was predominantly expressed in the adult brain and embryonic neural tissues. The inhibition of Brorin-like functions in zebrafish resulted in the impairment of neural development. Brorin-like potentially plays roles in neural development and functions.

1. Introduction

Bone morphogenetic proteins (BMPs) are secreted signaling molecules belonging to the TGF-β superfamily [1]. BMP genes are expressed in embryonic tissues and organs. It has become apparent that BMP signaling is critical for morphogenesis and development [2,3]. BMP genes are also expressed in developing neural tissues [4]. BMPs alter the fate of neural precursor cells from neurogenesis to astrocytogenesis [5,6]. Additional observations that BMPs are also continuously expressed in the adult brain [7–10] led us to the notion that BMPs may be involved in adult neurogenesis.

The functions of BMPs are regulated by secreted regulators. Many secreted BMP regulators have been described. In vertebrates, such proteins include Noggin, the Chordin family, Follistatin, FSRP, and the DAN/ Cerberus family [3]. We previously reported Brorin encoding a novel secreted BMP antagonist identified in mice and humans [11]. Brorin with two cysteine-rich domains is a member of the Chordin family [12]. The Brorin gene is predominantly expressed in embryonic and adult neural tissues, and Brorin protein promotes neurogenesis in cultured mouse neural precursor cells [11]. We also report here the identification of a novel secreted BMP antagonist, Brorin-like, in mice, humans, and zebrafish. The amino acid sequence of Brorin-like is similar to that of Brorin. Brorin-like protein inhibited the activity of BMP and promoted neurogenesis like Brorin. Mouse Brorin-like was predominantly expressed in the adult brain and embryonic neural tissues. The inhibition of Brorin-like functions in zebrafish resulted in the impairment of neural development. Brorin-like potentially plays roles in neural development and functions.

2. Materials and methods

2.1. Identification of Brorin-like in mice and humans

Amino acid sequences predicted from mouse cDNAs of unknown function in nucleotide sequence databases were randomly analyzed using PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences). cDNAs encoding putative secreted proteins were identified and cloned in a vector DNA, pBluescript II SK (+) (Stratagene). We named one of the cDNAs mouse Brorin-like. Human Brorin-like or zebrafish Brorin-like cDNA was also identified in a homology-based search of human or zebrafish cDNA sequences in nucleotide sequence databases, respectively.

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2.2. Forced expression of mouse Brorin-like cDNA in COS-7 cells and insect cells

The mouse Brorin-like cDNA with a DNA fragment encoding a Myc tag and a His6 tag at the 3’ terminus of the coding region was constructed between EcoRI and XbaI sites in a vector DNA, pcDNA3.1(+)(Invitrogen). COS-7 cells were transfected with the recombinant vector as described [11]. After the transfection, COS-7 cells were further cultured in fresh culture medium at 37°C for 72 h. Brorin-like in the culture medium and lysate of the transfected cells was detected by Western blotting using an anti-Myc tag antibody (Cell Signaling) as described [11].

The coding region of mouse Brorin-like cDNA with a DNA fragment encoding a Myc tag and a His6 tag at the 3’ terminus of the coding region was constructed in a transfer vector DNA, pAcGP67A. High Five cells infected with the recombinant baculovirus were cultured at 27°C for 72 h in serum-free medium EX-CELL 405 (JRH Bioscience). Mouse Brorin-like was purified from the culture medium by affinity chromatography using Ni-NTA agarose as described [11]. Purified mouse Brorin-like was analyzed by Western blotting using an anti-Myc tag antibody as described [11].

2.3. Alkaline phosphatase activity in MC3T3-E1 cells

MC3T3-E1 cells were treated with human BMP2 or BMP6 (R&D systems), mouse Noggin (R&D systems) and mouse Brorin-like for 72 h. Alkaline phosphatase activity in the cells was determined as described [11]. Results are the means ± S.E.M. for 12 independent wells from four independent experiments.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Mouse or zebrafish cDNA was transcribed from the RNA with Molony murine leukemia virus reverse transcriptase. Brorin-like or Brorin cDNA was amplified from the cDNA with Taq DNA polymerase and primers specific for mouse Brorin-like or Brorin or zebrafish Brorin-like. Mouse β-Actin cDNA or zebrafish elongation factor 1-α (ef1α) cDNA was also amplified with primers specific for mouse β-Actin or zebrafish ef1α as a control. The amplified DNA was analyzed by agarose gel (1.5%) electrophoresis.

2.5. In situ hybridization

Mouse embryos and brain at postnatal day 56 (P56) were cut at 16 μm with a cryostat, as described. The sections were examined in situ hybridization with a 35S-labeled mouse antisense Brorin-like or Brorin RNA probe as described [11]. Whole-mount in situ hybridization was performed using zebrafish embryos, as previously described [20]. Zebrafish Brorin-like or Brorin cDNA was cloned in a vector DNA, pCMT-T (Clonetech). DIGoxigenin-labeled Brorin-like and Brorin RNA probes were synthesized by in vitro transcription.

2.6. Neural differentiation activity in mouse neural precursor cells

Mouse neural precursor cells were prepared from the embryonic diencephalon at E13.5 as described [11]. The cells were treated with mouse Brorin-like or 10% FBS for 3 days. Neural differential activity in the neural precursor cell was determined by immunostaining using an anti-microtubule-associated protein 2 (MAP2) mouse monoclonal antibody (Sigma) or an anti-glial fibrillary acidic protein (GFAP) rabbit polyclonal antibody (Sigma) as described [11]. Results are the means ± S.E.M. for 20 different fields from four independent slides.

2.7. Morpholino oligonucleotide injection into zebrafish embryos

The sequences of Brorin-like and universal control MOs used were as follows: a splice-site-targeted Brorin-like MO (MO1), the antisense sequence (25 bases) of which corresponds to that between the first intron and second exon of the coding region, 5'-CCATTCAAGGCGAAGGCTGGG-3’; another splice-site-targeted Brorin-like MO (MO2), the antisense sequence (25 bases) of which corresponds to that between the first exon and intron of the coding region, 5'-CAGACAGGTGTCTTACCTTGAAC-3’; control MO (25 bases), 5’-CTCTTACCTCAGTTACAATTATA-3’. Brorin-like MO1 (2 ng), Brorin-like MO2 (4 ng) or control MO (2 or 4 ng) were injected into four central blastomeres of 16-cell embryos. To determine its efficacy, Brorin-like MO1 (8 ng) or Brorin-like MO2 (16 ng) was injected into 2-cell embryos. RNA was isolated from wild-type, Brorin MO1-injected or Brorin MO2-injected embryos at 24 hpf. Brorin-like cDNA and ef1α cDNA as a control were amplified from the RNA by RT-PCR, as described above.

2.8. Statistical analysis

Data are expressed as means ± S.E.M. The significance of differences in mean values was assessed using Student’s t-test.

3. Results and discussion

3.1. Identification of mouse and human Brorin-like

We identified mouse cDNA encoding a novel putative secreted protein of 222 amino acids with a putative signal sequence (21 amino acids) at its amino-terminus and two cysteine-rich domains in its core region (GenBank AB374230) (Fig. 1A). As the protein is significantly similar (48.6% amino acid identity) to mouse Brorin (324 amino acids), a secreted BMP antagonist [11], we named it mouse Brorin-like. Human Brorin-like cDNA (AB374231) was also identified. The amino acid sequence of human Brorin-like (222 amino acids) was highly similar (98.6% identity) to that of mouse Brorin-like (Fig. 1A).

3.2. Comparison of Brorin-like with members of the Chordin family

The Chordin family, a group of proteins with cysteine-rich domains that consist of 10 cysteine residues, comprises several members, Brorin, Chordin, Chordin-like 1/Neuralin/Neurogenesin-1 (CHRD1), Chordin-like 2 (CHRD2), and Cossyless-2 [11-16]. Brorin, Chordin, CHRD1, and CHRD2 are BMP antagonists; however, Cossyless 2 functions as a BMP antagonist and a pro-BMP factor. As Brorin-like is very similar to Brorin, Brorin-like is also a member of the Chordin family. Members of the Chordin family have different numbers (two, three, or four) of cysteine-rich domains (Fig. 1B). Brorin and Brorin-like have two sets of cysteine-rich domains, and other members have three or four sets, indicating that Brorin and Brorin-like are distinct from any other member of the Chordin family.

3.3. Forced expression of mouse Brorin-like cDNA in cultured cells, COS-7 cells

Mouse cDNA encoding Brorin-like was expressed in cultured cells, COS-7 cells. Both the culture medium and cell lysate were examined by Western blotting. Three major bands of ~40, 39, and 36 kDa were detected in the culture medium, indicating that Brorin-like is a secreted protein (Fig. 2A). The observed molecular masses were larger than the calculated molecular mass of the
recombinant Brorin-like protein (25,010 Da), indicating Brorin-like to be subjected to post-translational modification.

3.4. Production of purified mouse Brorin-like protein

To prepare purified mouse Brorin-like protein, mouse Brorin-like cDNA was expressed in cultured High Five insect cells by infection with a recombinant baculovirus containing the mouse Brorin-like cDNA. Mouse Brorin-like was purified from the culture medium by affinity chromatography. Purified Brorin-like was separated by SDS–polyacrylamide gel electrophoresis followed by protein staining. Purified Brorin-like showed a single band of 35 kDa with a band of bovine serum albumin (BSA) (66 kDa) as a carrier (Fig. 2 B). Purified Brorin-like also showed a single band of 35 kDa by Western blotting.

3.5. Effects of Brorin-like protein on BMP signaling in MC3T3-E1 cells

Brorin is a BMP antagonist, indicating that Brorin-like might be also. BMPs are signaling molecules for the stimulation of osteoblastic differentiation [17]. Most are members of the dpp and 60A subgroups of the TGF-β superfamily [4]. We examined the effect of mouse Brorin-like and mouse Noggin as a control of a BMP antagonist on the activity of BMP2 and BMP6, as representatives of the dpp and 60A subgroups, respectively, for the differentiation of
MC3T3-E1 cells by determining alkaline phosphatase activity [18]. Although the alkaline phosphatase activity stimulated by BMP2 or BMP6 was strongly inhibited by Noggin, the activity was weakly but significantly inhibited by Brorin-like (Fig. 3A). BMPs also induce the phosphorylation of Smad. BMP2 and BMP6 induced the phosphorylation of Smad1/5/8 in MC3T3-E1 cells. The phosphorylation induced by BMP2 or BMP6 was strongly inhibited by Noggin. However, the phosphorylation was not significantly inhibited by Brorin-like (data not shown).

3.6. Expression of Brorin-like in mouse embryos and adult tissues

The expression of Brorin-like in mouse embryos during E12.5–E18.5 was examined by in situ hybridization (Fig. 4Aa–d). Expression was significant in the developing neural tissues, including several discrete regions in the forebrain, midbrain, and hindbrain, and the spinal cord. The expression profiles of Brorin-like in embryos are similar to but distinct from those of Brorin (Fig. 4Ae–h) [11]. The expression of Brorin-like in mouse tissues at P56 was examined by RT-PCR. The expression of Brorin-like was predominantly detected in the brain among the major organs examined (Fig. 4B). The brain-specific expression profile is similar to that of Brorin (Fig. 4B) [11]. The expression of Brorin-like in the brain at P56 was also examined by in situ hybridization. Expression was widely detected in the brain (Fig. 4Ca–f). However, prominent expression of Brorin-like was observed in several discrete regions including the thalamus, hypothalamus, and hippocampus. The expression profiles of Brorin-like in the brain are also similar to but distinct from those of Brorin (Fig. 4Cg–l) [11]. These results indicate that Brorin-like has unique roles in developing and adult neural tissues.

3.7. Effects of Brorin-like on neuronal and astrocytic differentiation in cultured neural precursor cells

As the expression profiles of Brorin-like indicated its potential roles in neural differentiation, we examined the effect of mouse Brorin-like and FBS (10%), which promotes neural differentiation in neural precursor cells [19], as a control on the neural differentiation of mouse neural precursor cells. After the neural precursor cells had been cultured for 3 days, the differentiation was examined by double immunostaining using anti-MAP2 and GFAP antibodies for neurons and astrocytes, respectively. FBS increased the proportion of neurons and generated astrocytes among the neural precursor cells (Fig. 3B). Brorin-like also increased the proportion of neurons among the neural precursor cells. However, treatment with Brorin-like did not produce astrocytes. These results indicate that Brorin-like induced neurogenesis but not astrogenesis.

3.8. Expression and functions of Brorin-like in zebrafish embryos

As the functions of zebrafish genes can be effectively blocked by antisense morpholino oligonucleotides in vivo, the zebrafish is a useful model to elucidate the functions of genes in vertebrates in vivo [21]. We identified zebrafish DNA encoding Brorin-like (AB428728), of which the amino acid sequence (223 amino acids) is significantly similar (>78% identity) to that of mouse Brorin-like (data not shown). We examined the expression of Brorin-like in zebrafish embryos at 36 hpf by whole-mount in situ hybridization. The expression of Brorin-like was predominantly detected in the embryonic brain, including several discrete regions in the forebrain, midbrain, and hindbrain (Fig. 5Aa and b). The neural tissue-specific expression profile of Brorin-like in zebrafish embryos was also similar to but distinct from those of Brorin (Fig. 5Cg–l) [11]. These results indicate that Brorin-like has unique roles in developing and adult neural tissues.
is essentially consistent with that of Brorin-like in mouse embryos (Fig. 4A). We also identified zebrafish Brorin cDNA (AB292672) (unpublished data), and examined its expression. The expression profile of Brorin-like was similar to but slightly distinct from that of Brorin (Fig. 5Ac and d).

To inhibit Brorin-like functions in zebrafish embryos, we injected spliced-site-targeted Brorin-like MOs, MO1 and MO2, the sequences of which correspond to different boundary regions between the intron and exon of the coding region, into zebrafish embryos. We examined whether Brorin-like MOs could efficiently block the splicing of the Brorin-like mRNA precursor. The expression of mature Brorin mRNA examined by RT-PCR was markedly decreased in MO-injected embryos (Fig. 5B). These results indicate...
that Brorin-like MOs effectively blocked the maturation of Brorin-like mRNA. To examine the roles of Brorin-like in zebrafish neural development, we injected Brorin-like MO1 into four central blastomeres of 16-cell embryos. The MO1-injected embryos at 25 hpf showed morphological abnormalities in the brain and eye (n = 71/96) (Fig. 5Cc and d). In the Brorin-like knockdown brain, the formations of the telencephalic and tectal ventricles were apparently impaired. The formation of the constriction between the midbrain and hindbrain was also impaired. The Brorin-like MO2-injected embryos also showed similar morphological abnormalities (n = 20/32) (Fig. 5Cc and d). In contrast, universal control MO-injected embryos did not show any morphological abnormalities (data not shown). These results indicate that Brorin-like plays crucial roles in neural development. We also injected Brorin MO1 (a spiced-site-targeted MO) or MO2 (a translation initiation-site-targeted MO) into zebrafish embryos (unpublished observations). The Brorin knockdown embryos also showed morphological abnormalities in the brain and eye. However, the phenotype of Brorin knockdown embryos was apparently severer than that of Brorin-like knockdown embryos. The formations of the telencephalic and tectal ventricles and constriction between the midbrain and hindbrain were apparently impaired in the embryos. Furthermore, the embryos exhibited a significant reduction in the size of the brain (unpublished observations). As the phenotype of Brorin-like knockdown embryos is similar to but distinct from that of Brorin knockdown embryos, Brorin-like potentially plays roles which are similar to but distinct from those of Brorin, in neural development.

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