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Early specification of ascidian larval motor neurons

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Abstract

In the tadpole larvae of the ascidian *Halocynthia roretzi*, six motor neurons, Moto-A, -B, and -C (a pair of each), are localized proximal to the caudal neural tube and show distinct morphology and innervation patterns. To gain insights into early mechanisms underlying differentiation of individual motor neurons, we have isolated an ascidian homologue of *Islet*, a LIM type homeobox gene. Earliest expression of *Islet* was detected in a pair of bilateral blastomeres on the dorsal edge of the late gastrula. At the neurula stage, this expression began to disappear and more posterior cells started to express *Islet*. Compared to expression of a series of motor neuron genes, it was confirmed that early *Islet*-positive blastomeres are the common precursors of Moto-A and -B, and late *Islet*-positive cells in the posterior neural tube are the precursors of Moto-C. Overexpression of *Islet* induced ectopic expression of motor neuron markers, suggesting that *Islet* is capable of regulating motor neuron differentiation. Since early expression of *Islet* colocalizes with that of *HrBMPb*, the ascidian homologue of *BMP2/4*, we tested a role of BMP in specification of the motor neuron fate. Overexpression of *HrBMPb* led to expansion of *Lim* and *Islet* expression toward the central area of the neural plate, and microinjection of mRNA coding for a dominant-negative BMP receptor weakened the expression of these genes. Our results suggest that determination of the ascidian motor neuron fate takes place at late gastrula stage and local BMP signaling may play a role in this step.

Keywords: Ascidian; Motor neuron; LIM; BMP; Ion channel genes

Introduction

During animal embryogenesis, development of neuronal characteristics, such as morphology, axonal projection, membrane excitability, and neurotransmitter identity, depends on the timing and localization at which neurons arise. Among a variety of types of neurons, motor neurons are best documented for their function, morphology, and development both in vertebrates and invertebrates.

In the vertebrate spinal cord, the area for motor neuron generation is defined in the context of dorsoventral (D–V) patterning of the neural tube (reviewed in Tanabe and Jessell, 1996; Sasai and DeRobertis, 1997). This patterning is established by proteins secreted from overlaying ectoderm and the ventrally located notochord. Motor neurons are induced by a ventralizing factor Sonic Hedgehog (Shh), which is expressed in the notochord and subsequently in the floor plate of the neural tube (Roelink et al., 1994). A generation of dorsal phenotype of the neural tube involves other secretory factors, such as BMP (Delot et al., 1999; Leim et al., 1995, Neave et al., 1997). Vertebrate motor neuron precursors can be identified by the expression of *Islet*, a member of LIM type homeobox gene family

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(Ericson et al., 1992; Karlsson et al., 1990). *Islet-1* knockout mice fail to differentiate motor neurons, establishing that *Islet-1* is essential for vertebrate motor neuron differentiation (Pfaff et al., 1996). Several LIM type homeobox genes are expressed in the motor neurons and the combinatorial gene expression pattern of this family subdivides motor neurons into groups with different characteristics (Tokumoto et al., 1995; Tsuchida et al., 1994). The critical roles of LIM type homeobox genes in motor neuron differentiation have also been reported in *Drosophila* (Thor and Thomas, 1997; Thor et al., 1999).

The ascidian is a primitive chordate that belongs to a sister group of vertebrates. Anterior–posterior (A–P) patterning of the neural tube through a series of homeobox genes (Katsuyama et al., 1996, 1999) and the neural induction (Okado and Takahashi, 1988, 1993; Nishida, 1991; Inazawa et al., 1998) are both evolutionarily conserved features of ascidians when compared to vertebrates (Meinertzhagen et al., 2004). The CNS of swimming larvae of the ascidian consists of only several hundreds of cells, containing approximately 100 neurons (Nicol and Meinertzhagen, 1991). Despite of this structural and embryological simplicity of the ascidian larval CNS, the exact locations and cell lineage of individual motor neurons have not been defined until recently.

Our observations using lineage tracer and gene expression revealed that larval motor neurons are confined to a specific region of the neural tube that is at the proximal tail region (also called neck region; Okada et al., 2002). Three pairs of descendants of A5.2 blastomeres of the 16-cell stage embryo express neural markers in the proximal tail region of the larval CNS (Katsuyama et al., 2002) and all of these cells extend their axon to the muscle cells (Okada et al., 1997, 2002), indicating that all A5.2-derived neurons are motor neurons. On the other hand, A5.1 blastomeres give rise to putative interneurons, but not motor neurons. Unlike vertebrate spinal cord that contains motor neurons throughout its A–P axis, motor neurons in ascidian embryos are restricted to a part of the neural tube.

Location, morphology, and late development of individual motor neurons were investigated using two gene markers (Okada et al., 2002). One gene marker was *TuNa2*, which putatively encodes for a voltage-gated sodium channel (Okamura et al., 1994) and exhibits motor neuron-specific expression during embryogenesis (Nagahora et al., 2000; Okada et al., 2002). *TuNa2* expression was first detected as two pairs of spots in the lateral cells of the neural tube in the tailbud embryos (Nagahora et al., 2000; Fig. 2B). The anterior spot further divides into two spots during tailbud stage, resulting in three pairs of motor neurons that line along the A–P axis of the neural tube (Okada et al., 2002). The second gene marker was *Hrlim* (or *Lim*), an ascidian LIM type homeobox gene. This gene is expressed both in the motor neuron lineage and in the trunk region of the developing CNS (Okada et al., 2002; Wada et al., 1995; Fig. 2C). Individual motor neurons of *Halocynthia* larvae are designated to Moto-A, -B, and -C, and each has unique characteristics (Okada et al., 2002). Moto-A is most anteriorly located and its axon traverses dorsally toward the posterior end of the tail. Moto-B is the medial pair projecting their axon ventrally and extending it along the ventral muscle cells. It makes synapse only to a proximal region of the muscle bundle in the larval tail. Moto-C, the most posteriorly located pair, has an elongated cell body that projects its axon along the dorsal muscle band. However, it remains unknown how three pairs of motor neurons are specified at specific regions of the larval neural tube during embryogenesis.

Here, we isolated an ascidian homologue of *Islet* and examined regulation of its expression. Overexpression of *Islet* induced ectopic expression of motor neuron markers, which suggests that *Islet* regulates motor neuron differentiation. Comparison of the expression pattern of *Islet* with those of other genes suggests that *Islet* is expressed in all the precursors of three types of motor neurons. Overexpression experiments of *HrBMPb* and dominant-negative BMP receptor raise a possibility that expression of *Islet* and *Lim* in motor neuron precursors in the neural plate depends on BMP signaling.

Materials and methods

Cloning of ascidian Islet cDNA

The cDNA was reverse transcribed from polyA RNA of larvae of the ascidian *Halocynthia roretzi* using a random hexamer. A partial homeobox fragment was amplified from the cDNA. Specific primers used were AAYGARAARCARYTNCAYAC and TTCRCANCKYTTRTTYGRAACCA. A band of expected size was subcloned, sequenced, and found to be of a partial fragment of *Islet* cognate. This PCR fragment was used as a probe to screen *Halocynthia* larva cDNA library and four independent clones were isolated. All cDNA clones were sequenced completely. Two of the clones encoded for full-length Islet protein along with a partial stretch of polyA tail (accession number AB044142). These two clones showed almost identical sequence with only a few SNPs that had no effect on the amino acid sequence (data not shown). One clone lacked a 5′ side sequence along with polyA. Another had an insert of 23 bases between the nucleotide sequences coding for LIM domain and homeodomain, which might be an intron of the immature mRNA.

mRNA detection

Northern and in situ hybridizations were carried out as previously described (Katsuyama et al., 1995; Wada et al., 1995). The *Islet* probe for in situ hybridization was transcribed from ISL3a cDNA clone (Fig. 1; sequence accession number AB044142). Other probes for in situ hybridization were the same as those used previously (Katsuyama et al., 1999; Miya and Satoh, 1997; Okada et al., 2002).