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Gene Expression Patterns 7 (2007) 837–845

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The Slit receptor *Robo1* is predominantly expressed via the *Dutt1* alternative promoter in pioneer neurons in the embryonic mouse brain and spinal cord

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Received 17 May 2007; received in revised form 27 July 2007; accepted 31 July 2007
Available online 7 August 2007

Abstract

Robo1 is a member of the Roundabout (Robo) family of receptors for the Slit axon guidance cues. In mice (and humans), the *Robo1* locus has alternative promoters producing two transcript isoforms, *Robo1* and *Dutt1*. These isoforms have unique 5′ termini, predicted to encode distinct N-terminal amino acids, but share the rest of their 3′ exons. To determine the spatial expression of the *Robo1* and *Dutt1* isoforms, we generated isoform-specific RNA probes, and carried out in situ hybridization on E10.5 mouse embryos, the stage in early neuron differentiation when many major axon pathways are established. The two isoforms had distinct expression patterns that partially overlapped. *Dutt1* was the predominant isoform, with widespread expression in regions of post-mitotic neurons and neuroepithelial cells. The *Robo1* isoform had a distinct expression pattern restricted to subsets of neurons, many of which were *Dutt1*-negative. *Dutt1* was the main isoform expressed in spinal cord commissural neurons. For both probes, the main hybridization signal was limited to two spots in the nuclei of individual cells. This study shows distinct expression patterns for the *Dutt1* and *Robo1* alternative promoters in the embryonic nervous system.

Keywords: Axon guidance; Neuron; Robo; Slit; Longitudinal axon; Commissural axon; Dorsal root ganglion

1. Results and discussion

Growth cones navigate by selectively responding to molecular cues in their environment. Different types of axons differ in their responses to cues, depending on their expression of specific combinations of guidance receptors. In contrast to the great diversity in neuron types and their axon projection patterns, a surprisingly small number of axon guidance receptors have been identified to date. However, single receptor genes could potentially give rise to multiple receptor isoforms through mechanisms such as alternative promoters and differential mRNA splicing.

The Slit repellents and their Robo receptors are a major guidance system conserved from invertebrates to vertebrates (Dickson and Gilestro, 2006). The Roundabout (Robo) family of transmembrane receptors was first identified in Drosophila in a mutant screen for genes that control midline crossing by pioneer axons (Seeger et al., 1993). Slit/Robo signaling has a primary function in keeping axons out of the Slit + midline, both in commissural axons to prevent re-crossing, as well as in ipsilateral axons to prevent them from crossing at all. Robo homologs have been identified in mammals and other vertebrates (Kidd et al., 1998b; Sundaresan et al., 1998; Yuan et al., 1999). Robo1, Robo2, Robo3 (Rig1) and Robo4 are the four members found in human, rat and mouse. Vertebrate Robo expression is widespread, suggesting that Slit/Robo signaling has broad roles in guiding a diverse set of commissural, longitudinal, and other axon populations. For example, mice with *Robo* mutations have defects in midline crossing of spinal cord commissural axons (Long et al., 2004). Other axon populations dependent on Slit/Robo signaling include major forebrain tracts and commissures (Andrews

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doi:10.1016/j.modgep.2007.07.004

et al., 2006; Bagri et al., 2002), retinal axons (Plump et al., 2002; Thompson et al., 2006), and motor axons (Hammond et al., 2005), as well as others.

Recent evidence indicates that the small number of vertebrate *Robo* genes each produces more than one isoform. For example, alternative splicing of Robo2 and 3 transcripts results in isoforms with distinct affinity for Slit binding and/or differences in intracellular signaling (Camurri et al., 2004; Yue et al., 2006). Tissue-specific alternative mRNA splicing appears to contribute to the spatial complexity of Robo2 and 3 expression (Challa et al., 2005; Dalkic et al., 2006).

Robo1 presents a different case, with alternative transcription start sites providing the potential to be independently regulated. A human *Robo* homolog was independently cloned from breast and small cell lung cancer cell lines as a potential tumor suppressor gene and designated as *Dutt1* (Deleted in U-twenty twenty) (Sundaresan et al., 1998). Sequence analysis subsequently established that *Dutt1* cDNA sequences largely overlap with *Robo1*, but each has unique 5′-most exons and start codons. Therefore, *Robo1* and *Dutt1* genes are derived from alternative promoters. Interestingly, the *Robo1* and *Dutt1* promoters appear to have differential spatial and temporal transcriptional activity, based on RT-PCR analyses of several mouse tissues including developing brain (Clark et al., 2002). However, whether the *Robo1* and *Dutt1* isoform expression patterns are overlapping or distinct in the embryonic brain has not been determined. Intriguingly, one familial form of dyslexia has been correlated with a translocation breakpoint that causes *Robo1* haplo-insufficiency while leaving *Dutt1* transcription unaffected (Hannula-Jouppi et al., 2005). Together, these results suggest the potential for distinct patterns for each isoform.

To investigate the spatial expression from the two promoters for the *Robo1* and *Dutt1* isoforms, we generated isoform-specific probes for in situ hybridization, and used these to label the mRNA expression patterns of *Robo1* and *Dutt1* in mouse brain and spinal cord on embryonic day 10.5. E10.5 was chosen as a developmental stage in which several key types of brain stem and spinal cord neuron types were differentiating and extending axons, yet early enough to present a relatively simple spatial pattern of identified populations of neurons with diverse axon projection patterns.

1.1. Generation of isoform-specific RNA probes

In the structure of the mammalian *Robo1* locus, the first two exons, referred to as exon 1a and exon 1b, are unique to the *Robo1* isoform, while exon 2 is unique to *Dutt1* (Fig. 1A) (Clark et al., 2002). The isoforms share sequences from exon 3, and thus share the coding sequence for the Ig domains. The first 18 amino acids of Dutt1 correspond to a signal peptide (Fig. 1C). Robo1 extends further N-terminal with a 24 amino acid signal peptide followed by an isoform-specific 32 unique amino acid sequence. Previous studies showed tissue-specific differential expression of mouse *Dutt1*/*Robo1* isoforms with RT-PCR (Clark et al., 2002). Different transcription start sites indicate the possibility of promoters driving transcription in separate subsets of cells.

Our first aim was to identify the 5′ sequences unique to each mouse isoform. Sequences for the *Dutt1*-specific exon 2 were represented in mouse cDNA and EST databases. The longest exon 2 sequences were 1046 bp long, based on several independent sequences, suggesting that transcription from the *Dutt1* promoter produced an initial 5′UTR of 992 nucleotides and coding sequence of 54 nucleotides. Sequences for the *Robo1* isoform were more difficult to identify. Beginning with cDNA sequences from rat or human that corresponded to *Robo1*-specific exons 1a and 1b, BLAST searches identified conserved mouse genomic sequences for putative exons 1a and 1b. The first 218 bp of the predicted *Robo1* isoform consisted of exon 1a, containing 5′UTR (50 bp) and the beginning of coding sequence (72 bp), plus exon 1b (96 bp). However, no matches to exon1a and 1b could be identified in mouse cDNA or EST databases. Furthermore, BLAST searches using the shared exon 3 identified 96 ESTs, several with exon 2 sequences at their 5′ ends, but none with exon 1a and 1b sequence. Thus, *Dutt1* sequences are represented in the mouse EST databases, but not *Robo1*-specific sequences. One EST, AW494633, matched exon 3 sequence, but with a different sequence at its 5′ end that mapped to a BAC-derived genomic sequence 360 kb upstream of exon 2 (lying between the putative exon 1a and 1b). This suggested a third promoter also poorly represented in EST databases, and was not further characterized.

To verify that the *Dutt1* and *Robo1* mRNAs were present in mouse embryos, and to isolate cDNAs sufficiently long for in situ hybridization probes, we used RT-PCR to amplify *Robo1* and *Dutt1* fragments with exon-specific primers from a cDNA pool from whole E10.5 mouse embryos (Fig. 1B). Fragments of the predicted sizes were produced for each isoform, 559 bp for *Dutt1* and 200 bp for *Robo1*, suggesting that the predicted exons were in fact transcribed. Reverse primers included T7 minimal promoter sequences to allow synthesis of anti-sense riboprobes. $Dutt1^{ex2}$ indicates a *Dutt1*-specific probe while $Robo1^{ex1a+1b}$ corresponds to a *Robo1*-specific probe. For a common probe to recognize both *Robo1* and *Dutt1* mRNA, we used the previously published rat cDNA subclone that spans the sequence encoding the first three Ig domains (Kidd et al., 1998b), and refer to this as the $Robo1^{ex3}$ probe.

1.2. Robo1 and Dutt1 are differentially expressed in the embryonic brain

To gain an overview of the expression patterns of *Robo1* and *Dutt1* in developing mouse embryos, we performed whole mount in situ hybridization on E10.5 (Fig. 2). This