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Gene expression pattern

Neuralin-1 is a novel Chordin-related molecule expressed in the mouse neural plate

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Abstract

Cysteine-rich repeats (CRs) of the type described in Chordin constitute conserved domains present in an expanding family of secreted molecules. These motifs were shown to mediate directly the antagonism of BMP signaling by Chordin and play a major role during development. Here we report the cloning and expression pattern of *neuralin-1*, a new member of the *chordin* family. The mouse cDNA was cloned by homology with a human genomic sequence encoding putative CRs. In the human genome, *neuralin-1* transcripts are encoded by 8 exons that span a region of at least 80 kilobases located on chromosome Xq22.1-23. Neuralin-1 is a 333 amino acid protein containing three CRs, two of them highly similar to the Chordin CRs that bind BMP. Like *chordin*, *neuralin-1* is able to induce secondary axes after mRNA injection in *Xenopus* embryos. Interestingly, during late gastrulation, *neuralin-1* and *chordin* present distinct and complementary expression patterns in the mouse: *neuralin-1* expression starts in the neural plate at mid-gastrulation, whereas *chordin* expression at that stage is restricted to the node and midline mesendoderm. Later on, *neuralin-1* expression becomes restricted to discrete regions of the central nervous system and to derivatives of the neural crest cells. During organogenesis, *neuralin-1* presents a broad expression pattern in many tissues such as dorsal root ganglia, gut, condensing cartilages of the skeleton and developing hair follicles. © 2001 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Neuralin-1; Chordin; CR domain; BMP; Extracellular matrix; Neural plate; Organogenesis; Forebrain; Neural crest; Dorsal root ganglion; Branchial arch; Cartilage; Axial skeleton; Intestine; Lung; Kidney; Hair follicle

1. Results

BMP signaling is tightly regulated in the extracellular space by binding to antagonists such as Chordin or Noggin. In the case of Chordin, it was shown that the inhibition is due to the binding of BMP to particular domains named Cysteine-rich repeats (CRs) (Larrain et al., 2000). These domains are characterized by the conservation of ten cysteines, including the carboxy-terminal motif CCXXC. The family of Chordin-related molecules has been recently expanded with the identification of new extracellular proteins containing multiple CRs, such as Nel-1 (Matsuhashi et al., 1995), CRIM1 (Kolle et al., 2000), Crossveinless-2 (Conley et al., 2000) and Kielin (Matsui et al., 2000) that contain 4, 5, 6 and 27 CRs, respectively. Based on the conservation of CR sequences and thanks to the recent development of genomic databases, we report the cloning and expression pattern of a novel member of the Chordin family.

Using BLAST search (Altschul et al., 1990), we found three predicted CRs as part of a large genomic BAC sequence located on human chromosome Xq22.1-23, generated by the Sanger Centre (GenBank AL049176; Fig. 1A). Using these human sequences, we amplified by PCR a specific 150 nucleotide fragment from mouse embryonic day 7.5 (E7.5) cDNA. This fragment was used to screen an arrayed cDNA library from mouse E12.5 embryo and led to the isolation of a 3.8 kb clone. This full-length clone is composed of an 1 kb ORF followed by a 2.6 kb 3′UTR (accession number AF305714). Comparing the mouse cDNA sequence with the human genomic sequence, all coding exons could be mapped including exon 6 that was not predicted by genome sequencing (Fig. 1A,B). We also identified the first exon of the human gene in an overlapping genomic clone (GenBank AC022687). Several mouse ESTs corresponding to *neuralin-1* 3′UTR indicate its expression in the mammary gland (AI036402), neonate skin (BB008656), olfactory brain (BB048228), cartilage (AV290589) and epididymis (AV379546).

The *neuralin-1* cDNA encodes a 333 amino acid protein containing a signal peptide and three CRs (Fig. 1B,C). It shares 96% amino acid identity with its human homologue.

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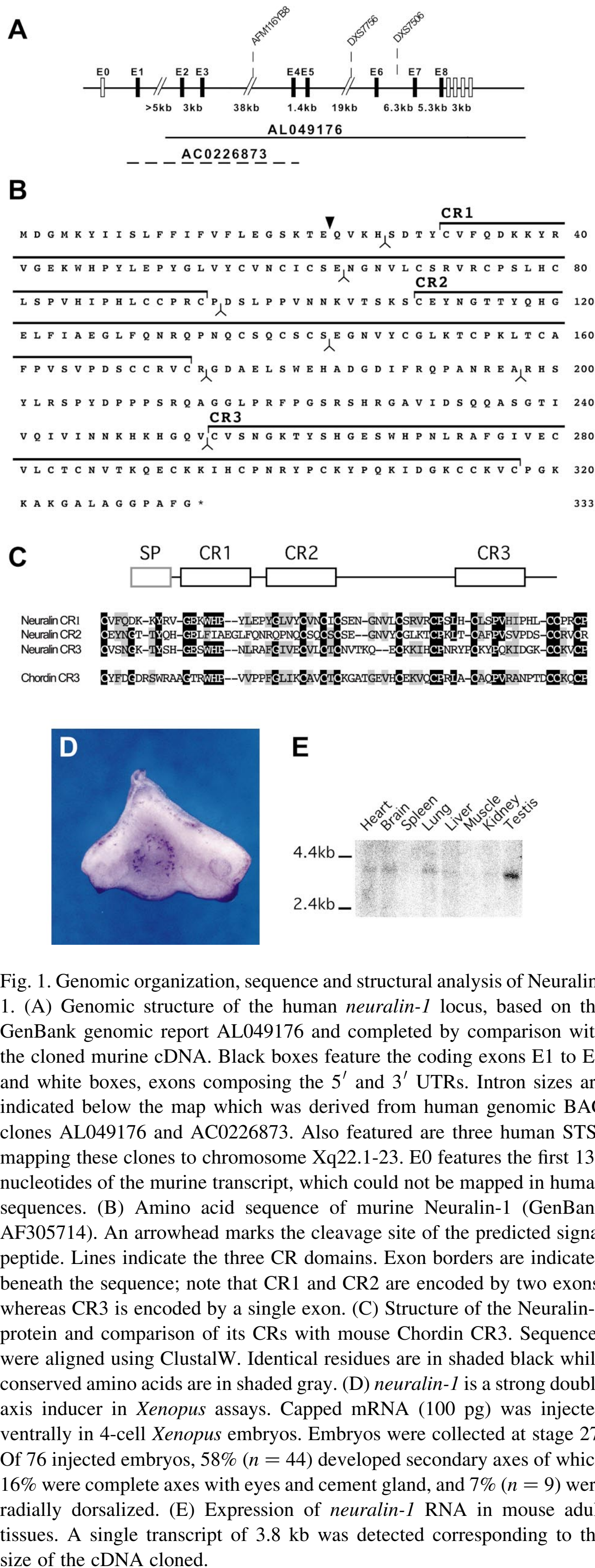

Fig. 1. Genomic organization, sequence and structural analysis of Neuralin-1. (A) Genomic structure of the human *neuralin-1* locus, based on the GenBank genomic report AL049176 and completed by comparison with the cloned murine cDNA. Black boxes feature the coding exons E1 to E8 and white boxes, exons composing the 5′ and 3′ UTRs. Intron sizes are indicated below the map which was derived from human genomic BAC clones AL049176 and AC0226873. Also featured are three human STSs mapping these clones to chromosome Xq22.1-23. E0 features the first 130 nucleotides of the murine transcript, which could not be mapped in human sequences. (B) Amino acid sequence of murine Neuralin-1 (GenBank AF305714). An arrowhead marks the cleavage site of the predicted signal peptide. Lines indicate the three CR domains. Exon borders are indicated beneath the sequence; note that CR1 and CR2 are encoded by two exons, whereas CR3 is encoded by a single exon. (C) Structure of the Neuralin-1 protein and comparison of its CRs with mouse Chordin CR3. Sequences were aligned using ClustalW. Identical residues are in shaded black while conserved amino acids are in shaded gray. (D) *neuralin-1* is a strong double axis inducer in *Xenopus* assays. Capped mRNA (100 pg) was injected ventrally in 4-cell *Xenopus* embryos. Embryos were collected at stage 27. Of 76 injected embryos, 58% ($n = 44$) developed secondary axes of which 16% were complete axes with eyes and cement gland, and 7% ($n = 9$) were radially dorsalized. (E) Expression of *neuralin-1* RNA in mouse adult tissues. A single transcript of 3.8 kb was detected corresponding to the size of the cDNA cloned.

No additional structural domains were detected using PROSITE (Hofmann et al., 1999). Neuralin-1 CRs present the characteristic spacing of cysteines present in most CRs including the carboxy-terminal motif CCXXC (Fig. 1B). Additional conserved residues such as the triad tryptophan-histidine-proline (WHP) render this homology particularly interesting as they are also present in Chordin CR1 and CR3 (Fig. 1C) which are known to bind BMP (Larrain et al., 2000). This observation is consistent with results obtained after *neuralin-1* mRNA injection into *Xenopus* embryos. *neuralin-1* was able to induce axis duplication or strong dorsalization (Fig. 1D). The frequency of complete axis with eyes and cement gland was higher than in the case of *chordin* (Sasai et al., 1994).

In situ hybridization analyses on mouse embryos revealed that *neuralin-1* expression started at neural plate stage (Fig. 2A). This expression extended to the entire neural plate at late headfold stage (Fig. 2B,C). Interestingly, at that time, *chordin* expression exhibits a complementary pattern and is restricted to the node (Bachiller et al., 2000). By E8.5–E9, neural expression became restricted to discrete regions of the forebrain and midbrain, as well as in the closing posterior neural folds (Fig. 2D,E). Starting at E8.5, *neuralin-1* expression was observed in a population of neural crest cells that migrate from the hindbrain and colonize the branchial arches (Fig. 2D–F). In addition, the dorsal root ganglia, another neural crest derivatives, were positive for *neuralin-1* expression (Fig. 2E,F).

During organogenesis, *neuralin-1* was expressed in a complex pattern (Fig. 3). At E13, expression in the nervous system was observed in dorsal root ganglia and in superficial layers of the CNS (Fig. 3A,C). *neuralin-1* transcripts were observed in mesenchymal cells throughout the embryo, including the forming cartilages of axial structures, ribs and facial skeleton (Fig. 3A,B,D). In addition, *neuralin-1* expression was detected in mesenchymal compartments of many organs such as the intestine, lungs, kidneys and the dermal papillae of the vibrissae follicles (Fig. 3E–H). This expression pattern coincides with sites of inductive processes involving the BMP signaling pathway during organ formation. In the adult, *neuralin-1* expression was detected in the heart, brain, lung, liver, kidney and testis as shown in Fig. 1E.

In conclusion, we have isolated a new member of the Chordin family. The expression of this CR-containing molecule and its inductive properties in *Xenopus* embryos make it a good candidate to modulate the BMP signaling pathway during formation of the neural plate. Several human hereditary diseases, including several types of X-linked mental retardation, map to the Xq22.1-23 region where the *neuralin-1* locus resides (www.ncbi.nlm.nih.gov/genome/guide/HsChrX.shtml).

2. Methods

A full-length cDNA was isolated from a Rapid-Screen arrayed cDNA library (OriGene) made from E12.5 mouse embryos. The library was screened by PCR using the follow-

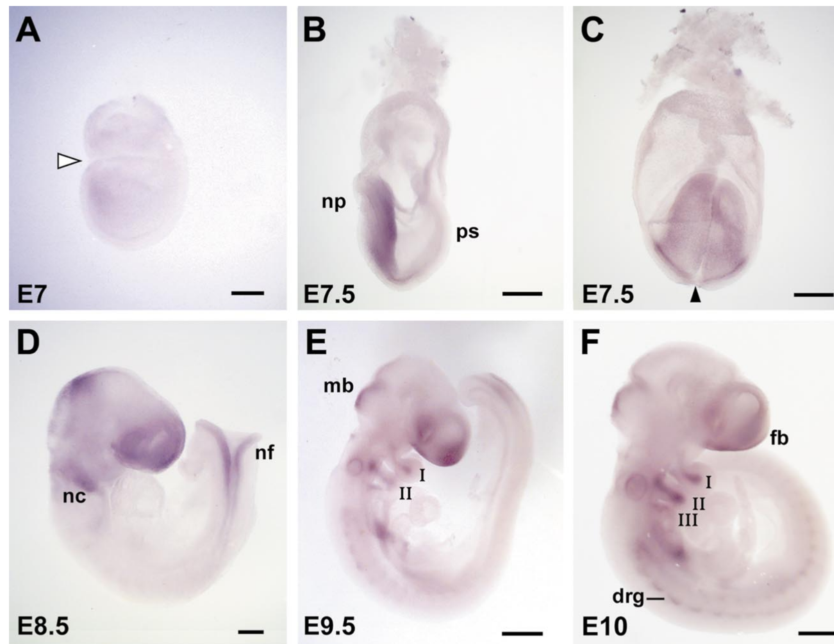

Fig. 2. Expression of *neuralin-1* in E7.5 to E10 mouse embryos by whole-mount in situ hybridization. (A) Neural plate stage embryo. Weak staining is detected in the forming neural plate. The arrowhead indicates the anterior embryonic-extraembryonic border. (B) Lateral and (C) frontal views of a late headfold stage embryo. *neuralin-1* is expressed in the entire neural plate (np); ps; primitive streak. The arrowhead indicates the node in which *chordin* is expressed at that stage. Note that *neuralin-1* is not expressed in the node and therefore has a complementary pattern to that of *chordin*. (D) E8.5 embryo. The anterior neural plate staining is now restricted to the forebrain and the dorsal midbrain. Two additional expression domains are observed in the neural crest (nc) migrating from the hindbrain and in the posterior neural folds (nf). (E) E9.5 and (F) E10 embryos. The branchial arches are stained (I–III) as well as the dorsal root ganglia (drg), both are derivatives of the neural crest. mb: midbrain, fb: forebrain. Scale bars represent: (A–C) 150 μm; (D) 250 μm; (E,F) 500 μm.

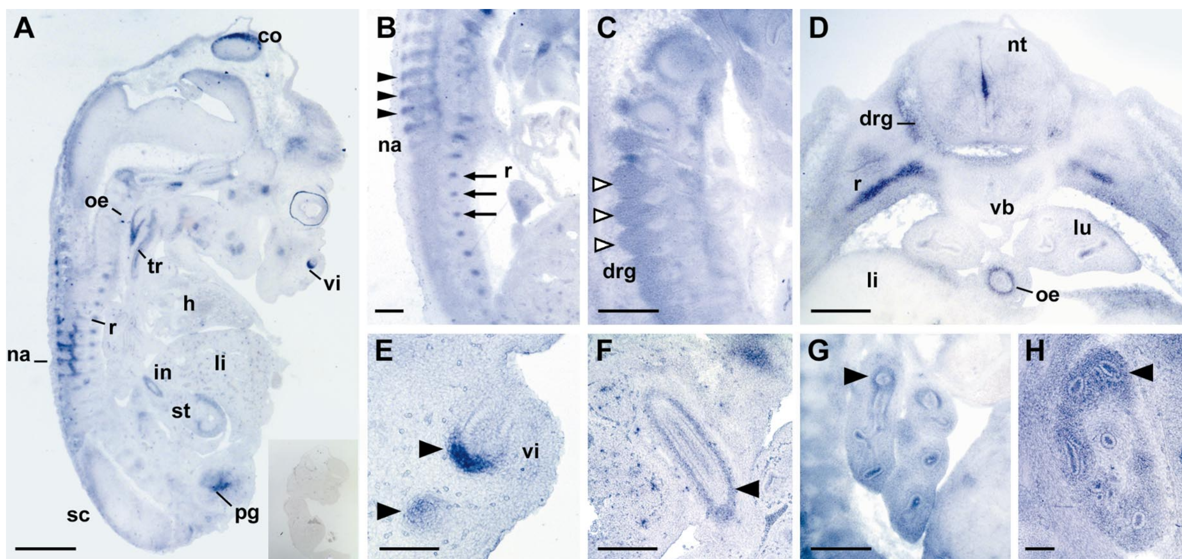

Fig. 3. Expression of murine *neuralin-1* at stage E13 detected by in situ hybridization on sections. (A) Sagittal section showing expression in condensing cartilages of the face, in axial structures such as the neural arches (na) of the vertebrae, the ribs (r) and the pelvic girdle (pg). *neuralin-1* expression is also detected in the superficial layer of the cortex (co) and spinal chord (sc), and in the mesenchyme surrounding endodermal derivatives such as the intestine (in), stomach (st), trachea (tr), esophagus (oe). Inset: hybridization negative control using a sense probe; h: heart, li: liver, vi: vibrissae. (B,C) Parasagittal sections showing *neuralin-1* expression in the forming axial skeleton. Arrows: ribs (r); black arrowheads: neural arches (na); white arrowheads: dorsal root ganglia (drg). (D) Transverse section through the trunk. drg, dorsal root ganglion; li, liver, lu, lung; nt, neural tube, vb, vertebral body; oe, oesophagus; r, rib. Note the strong expression in the rib cartilage and in the oesophagus mesenchyme. (E) *neuralin-1* expression in the mesenchyme (arrowhead) located at the tip of the vibrissae follicles (vi); this region later develops into the permanent portion of the hair follicles. (F–H) Detailed views of *neuralin-1* expression in the mesenchyme (arrowheads) of the intestine, lung and kidney respectively. Scale bars represent: (A) 1 mm; (B,C,G) 1.5 mm; (D,F,H) 2 mm; (E) 200 μm.

ing primers, 5′- AAG TAC AGA GTG GGT GAG AGA TGG CAT CC -3′ and 5′- CTC TGA CTA CCC GGC AGC AGG AAT CTG G -3′. These amplified a 150 nucleotide fragment containing *neuralin-1* CR1 and CR2. A 2.5 kb *Eco* RI fragment containing the entire open reading frame was subcloned into pCS2+ and sequenced on both strands. The pCS2-*neuralin-1* vector was also used as template to generate capped RNA. mRNA (100 pg) was injected ventrally in *Xenopus* embryos at the 4-cell stage (Sasai et al., 1994). Northern blot analysis on mouse adult tissues using a P^{32} random-labeled probe, was carried out on a mouse Multiple Tissue Northern Blot supplied by Clontech, which contained 2 μg of $poly(A)^+$ RNA per lane. Digoxigenin anti-sense probes were synthesized using the same 2.5 kb fragment subcloned into pKS^+, linearized with *Not* I and transcribed using the T3 RNA polymerase. Embryos were staged according to Downs and Davies (1993). In situ hybridizations were realized as described for whole-mount (Belo et al., 1997), and for sections (Henrique et al., 1995). Control sense probe did not detect any signals (see inset Fig. 3A).

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